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(57) Abstract

A series of new bacterial strains which are particularly useful for vaccine manufacture, for the treatment of intestinal and similar diseases such as cholera. The invention also includes a new method of Genetic Engineering particularly useful for manufacture of the vaccines. Bacterial strains are characterised by having a gene set substantially derived from a non-pathogen or a pathogen rendered non-pathogenic and able to invade, multiply, and briefly colonize the lymphoid tissue of the small intestine of mammalian species and having an introduced gene, genes or part thereof effective to specify synthesis of an antigen or antigens effective to generate a protective immune response due to production of IgA antibodies into the small intestine and other secretory surfaces.

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BACTERIAL STRAINS.

This invention relates to bacterial strains which are useful and which are achievable by genetic engineering techniques.

The problem to which this invention at least in its broadest sense can be said to relate is a difficulty associated with providing for effective immunisation against a major category of intestinal pathogens.

Such pathogens can include and are typified by Vibrio cholerae.

Bacterial intestinal pathogens fall into two categories. In the first are those which cause their effects by producing a toxin acting on the intestinal epithelial cells whilst the organism itself is confined to and grows in the intestinal lumen: Vibrio cholerae, which causes cholera in man, and enterotoxigenic Escherichia coli, different strains of which cause diarrhoea in man, swine scours and calf scours all belong to this category. In the other division are organisms like Salmonellae which in both man and animals can cause systemic disease in which diarrhoea may be an important component. These organisms penetrate through the wall of the intestine initially into the lymphoid tissue there, known as Peyer's patches; during the incubation phase they grow inside these sites and may either be eliminated as a subclinical infection or may lead to overt disease during which they may spread through the bloodstream beyond the intestinal sites.

Those organisms in the first category can be resisted by hosts possessing specific antibodies if these

are secreted into the intestinal tract and indeed recovery from infection is usually accompanied by reasonable amounts of intestinal antibody for short periods. With Salmonellae possession of specific antibody is by itself not sufficient to confer immunity; other cellular changes must occur as well. Nevertheless, probably due to their ability to multiply and colonise Peyer's patches, these organisms provoke much better antibody responses of the secretory IgA type than any of those of the former, non-penetrating kind. Using animal models, we have shown that hybrid organisms which possess basically the Salmonella genome plus a small piece specifying the antigenic structure of organisms of the second category behave fundamentally like native Salmonellae when given by mouth: they penetrate and colonise Peyer's patches temporarily and stimulate very good local IgA antibody responses measurable in the intestinal fluid: this antibody response includes one against the introduced antigen.

Vaccine strains according to this invention therefore combine the immunogenic potential of organisms of the first category typified by Samonellae, with the desired antigens of organisms of the second category, typified by Vibrio cholerae.

Hitherto, with the notable exception of live oral polio vaccine most effective vaccines which have been used and proposed involve the intramuscular route.

With reference to Vibrio cholerae, a mutant strain, Texas Star-SR, U.S. Patent 4328290, and similar genetically engineered strains have been proposed for production of live attenuated vaccines which can be

administered orally. These strains, however, suffer from certain drawbacks not the least of which is the fact that they cause limited diarrhoea even with all the known toxin genes deleted. Explanations for this residual diarrhoea are that it is caused by other virulence determinants of V. cholerae associated with the ability of these organisms to colonize the epithelium of the small bowel or another toxin. This being so the presence of some residual toxicity might be the price to pay for the use of such attenuated strains of enteric organisms if they are to remain effective immunizing agents. In one case of Vibrio cholerae some of this residual toxicity may be due to the extracellular haemolysin it produces.

After substantial investigation, we have found that there is advantage in consideration of using a vaccine appropriate for the oral route and by providing through genetic engineering techniques characteristics of a live bacterial strain that will firstly invade, multiply and briefly colonise the lymphoid tissue of the small intestine of mammalian species, and through an introduced gene or genes or parts thereof effect synthesis of an antigen or antigens to produce IgA antibodies, that such an approach has now been found to enable the production of very effective vaccines.

Accordingly, the invention can be said to reside in a bacterial strain characterised by having a gene set substantially derived from a non pathogen or a pathogen rendered non pathogenic and able to invade, multiply and briefly colonise the lymphoid tissue of the small intestine of mammalian species, and having an introduced gene or genes or parts thereof effective to specify synthesis of an antigen or antigens effective to induce production of IgA antibodies into the small intestine and other secretory surfaces.

The lymphoid tissue is typified by the Peyer's patches in the small intestine.

Preferably, the antigen or antigens are such as to induce production of IgA antibodies effective against any of the following genera where this is a pathogen; Escherichia, Rotavirus, Vibrio, Aeromonas and Bordetella.

With the exception of Bordetella, all pathogens of the above listed genera are enteric organisms. All are characterized by a mechanism of pathogenesis which involves the prerequisite step of mucosal adherence followed by toxin production, that is, they are not of the penetrating type. Although Bordetella is not a pathogen of the gut, but of the lungs, this invention can provide for protection against pathogens which adhere to any secretory surface. This is because there exists a central pathway by which antigenic stimulation of IgA precursor cells in the Peyer's patches results in the dissemination of sensitized cells to distant mucosal sites, such as the lungs where they can provide effective immunological protection.

A further aspect of this invention is that the stimulation of IgA production provides a means of measuring the immune response generated. Should the introduced genes code for a protective antigen of an invasive organism such as Campylobacter, Salmonellae or Shigella, the production of IgA antibodies directed against the introduced antigen will provide a means of measuring the generated immune response even though the IgA antibodies are not the important protective factor in these cases.

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The introduced gene or genes can be either within the chromosome of the bacterial strain or they can be on a plasmid within the bacterial strain.

Preferably, the pathogen rendered non pathogenic is selected from any one of the following, namely, Escherichia, Salmonella or Shigella.

Preferably, the introduced gene or genes specify the synthesis of at least part of the following, namely adhesins, outer membrane proteins or lipopolysaccharides of the pathogen.

Preferably, in one case, the gene or genes specify the synthesis of the K88 antigen of an enterotoxigenic Escherichia coli.

Preferably, as an alternative, the introduced genes specify the synthesis of the K99 antigen of an enterotoxigenic Escherichia coli.

Preferably, as an alternative, the introduced gene or genes specify the synthesis of one or more of the antigens CS1, CS2 or CS3 of a CFA/II producing enterotoxigenic Escherichia coli.

In an alternative arrangement, the genetic material specifies the synthesis of the colonisation factor antigens of enterotoxigenic Escherichia coli pathogenic to man.

Preferably, as a further alternative, the genetic material resides in any one of the plasmids pPM440, pPM500, pPM510 or pPM455, which encode genes specifying outer membrane proteins of Vibrio cholerae.

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In a further alternative, the introduced genes are effective to synthesize an O-antigen of Vibrio cholerae.

The synthesis of an O-antigen of Vibrio cholerae has been achieved but has involved particular difficulties which will be discussed later in the specification.

Preferably, the synthesized O-antigen of Vibrio cholerae is present on the surface of Escherichia coli.

As an alternative, the synthesized O-antigen is present on the surface of Salmonella or Shigella.

Further to the above, it has been observed that some avirulent Salmonellae strains have retained their capacity to invade the lymphoid follicles (Peyer's patches) following oral administration.

Indeed, it has been found that there is a direct correlation between the degree and length of colonisation of the lymphoid follicles by respective Salmonellae strains and their ability to stimulate immunity (refer to Table 1).

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TABLE 1

Immunising Strain	Kaufmann White Antigens	Survival in* Peyer's patches	Intestinal# IgA/ug/ml	%survival challenge S. typhimuriu
(S. typhimurium/				
E. coli hybrid 885	08	+	75	90
S. salford	16	+	38	90
S. strasbourg	9,46	+	10	90
S. budapest	1,4,12	+	5	42
S. typhimurium	1,4,5,12	-	2.5	27
S. fridenau	13,22	-	2.5	10
(E.coli/ S. typhimurium) hybrid 1142	4,5,12	-	2.5	10

* 10^3 organisms on day 7 after feeding 5 x 10^8 bacteria

\# Against the homologous lipopolysaccharide antigens.

The carrier organism can be typified by galactose epimeraseless Samonellae, or virulence-plasmidless Shigellae.

The extent of avirulence is one factor only but another of importance is that the strain be able to invade the Peyer's patches in sufficient numbers and once invaded, their colonisation should be limited to only a few days, typically 3 to 10 days. The result of this will be normally that the host will actively mount a long lived immune response which will also clear the organism from the host.

An additional advantage of all these vaccines is that they are simultaneously protective against the bacterial species which is used as the carrier.

Other advantages of bacterial strains, constructed according to this invention and used as the active ingredient in live, oral vaccines are that a single or limited number of doses will be effective, the vaccine strain will be well characterized genetically with no theoretical possibility of reversion to virulence, and the vaccine is suitable for use in both an active and passive immunization program as the IgA antibodies produced can be passed from mother to infant in colostrum and milk.

EXAMPLE 1

A live bacterial strain useful as an oral vaccine against scours (enteric colibacillosis) in swine.

One useful vaccine according to this invention is a Salmonella typhimurium galactose epimeraseless mutant harbouring a plasmid expressing the K88 antigen of an enterotoxigenic Escherichia coli. Such a strain is a

useful immunogen to induce the production of gut antibodies to the K88 pilus resulting in protection against the enterotoxigenic E. coli K88 strain.

It has been shown that vaccination of a sow with a purified K88 preparation can confer on her offspring passive immunity to a K88 E. coli. It could be concluded that antibodies to the K88 antigen in colostrum and milk protect the piglets by neutralization of the adhesive properties due to the K88 antigen. Thus, antibodies to the K88 adherence factor; the K88 pilus, will protect. Other adhesin type antigens designated, K99 987P, F41 have also been identified as important in piglet enteric colibacillosis. These, like the K88 adhesin could be used in the construction of a vaccine as demonstrated here for K88 pilus antigen.

We have used Salmonella typhimurium strain G30 (a galE mutant) as the carrier strain.

Plasmid pFM 205 which encodes the cloned K88 antigen from enterotoxigenic E. coli has been introduced into strain G30. Mice were then immunized by feeding 10^8 live bacteria or injecting intraperitoneally 5×10^7 formalin killed bacteria. Immunization was performed 3 times at weekly intervals. After one month the mice were bled and then sacrificed and their intestines removed and washed. The levels of antibodies to K88 were determined by ELISA using purified antigen (homogeneous by polyacrylamide gel electrophoresis). The results show (Table 2) that oral immunization produced a level of serum antibodies similar to that produced by intraperitoneal immunization. In contrast no detectable antibodies were produced in the gut when the bacteria were injected intraperitoneally whereas good gut antibody levels were induced as a result of oral immunization.

TABLE 2

Antibody production against purified K88 pili

Immunization	ELISA Titre	
	Serum	Gut washings
G30 orally	< 256	< 256
" intraperitoneally	< 256	< 256
G30/pFM205 orally	32768	4096
" intraperitoneally	131071	< 256
Normal mouse serum	< 256	

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EXAMPLE 2

A live bacterial strain effective for protection against a neonatal diarrhoea due to enterotoxigenic K99 E. coli for calves, lambs and piglets.

The introduction of a plasmid harbouring the K99 pilus, pRI9906-1 for example, into strain G30 is also another useful vaccine according to this invention. Such a vaccine would protect calves, lambs and piglets against neonatal diarrhoea due to enterotoxigenic K99 E. coli.

EXAMPLE 3

The introduction of plasmids harbouring the colonization factor antigens (CFA) of enterotoxigenic E. coli pathogenic for man would be further examples according to this invention. Such vaccines would protect against traveller's diarrhoea produced by enterotoxigenic E. coli of the homologous type.

Several known colonization factors have been described in enterotoxigenic E. coli: CFA/I, CFA/II, CFA/III and E8775. These antigens each represent a single fimbrial type with the exception of CFA/II where three possible surface antigens may be produced depending upon the biotype of the strain. These three

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designated CS1, CS2 and CS3, CS1 and CS3 are expressed in strains of the A biotype and CS2 and CS3 in the biotypes B, D and F. The genes for CS3 have been cloned in a series of plasmids of which pPM474 has been analyzed in detail and represents the type (Figure 20). Contiguous DNA has also been cloned as in plasmids pPM476, pPM477 and pPM483 and these are thought to encode at least CS1 and possibly CS2 in addition to CS3.

Transposon insertion analysis of pPM474 has shown that a region of at least 3.5 kb is responsible for CS3 production (Figure 21). This implies that it is a genetically complex process since the amount of DNA required to encode a protein the size of the subunit is only about 500 base pairs. Evidence that plasmids containing contiguous DNA encode at least CS1 in addition, comes from the fact that a spontaneous deletion of the wild type CFA/II plasmid which had lost the ability CS1 and CS3 had deleted about 15 kb. The end of the deletion corresponds to the 0.71 kb fragment on the right end of our cloned DNA. Thus both CS1 and CS3 are encoded within 15 kb to the left, and we have cloned about 16 kb (see Figure 20).

An example of a particular useful vaccine according to this invention is the introduction of plasmid pPM474 harbouring the CS3 antigen of CFA/II into S. typhi strain Ty21a.

EXAMPLE 4

This now describes a useful bacterial strain useful as an oral vaccine against Vibrio cholerae.

The currently available parenteral killed vaccine against cholera is virtually useless. Firstly, the bacteria have been killed which is likely to damage or destroy a variety of surface antigens which may be very important. Secondly, the route of immunization is not appropriate for the generation of antibodies of the IgA class on the mucosa of the gut. Thus, we have set about developing a superior live vaccine harbouring the antigens of importance and which can get to the optimal site of mucosal antibody (Iga) stimulation, the Peyer's patches.

The vaccine, a further example of this invention, is a live bivalent oral vaccine, being simultaneously protective against Salmonella typhi and Vibrio cholerae. The vaccine is based on a galactose epimeraseless (galE) derivative of the Salmonella typhi strain Ty2. Such a mutant S. typhi, namely Ty21a, has been shown to be a safe live oral vaccine capable of inducing protection against typhoid fever. Its preparation and properties are described in U.S. Patent No. 3,856,935 to R. Germanier. This aspect of the invention involves the cloning, by recombinant DNA or genetic engineering techniqus, of surface antigens of Vibrio cholerae and the introduction of these antigens into a S. typhi galE strain to produce a genetic hybrid. The hybrid is essentially the S. typhi galE strain containing and expressing the DNA for specific antigens of Vibrio cholerae.

The surface antigens of Vibrio cholerae upon which this vaccine is based fall into two classes, either the lipopolysaccharide (LPS) or surface proteins. These antigens are referred to as protective or target antigens in that they can induce the production of antibodies which will protect against Vibrio cholerae.

Cloning of the protective antigens

Vibrio cholerae strains 569B (a Classical Inaba strain) and 017 (an E1 Tor Ogawa strain) have been used as the source of donor chromosomal DNA for the various cloning experiments. This DNA was partially digested with one of the restriction endonucleases Sau3A, BamH1 or Pst1.

The vector DNA, pBR322, was digested with BamH1 (for cloning either Sau3A or BamH1 digested chromosomal DNA) or Pst1 and subsequently treated with alkaline phosphatase to prevent self-ligation. The two DNAs were mixed, ligated overnight, and transformed into a restrictionless E. coli K12 strain. Transformants were selected on nutrient media containing either ampicillin (when using BamH1 digested pBR322) or tetracycline (when pBR322 had been digested with Pst1). Such experiments resulted in greater than 80% of the transformants containing chromosomal inserts in the vector.

The transformants were patched onto numbered grids on nutrient medium containing the appropriate antibiotic and grown up overnight at 37°. They were then transferred to nitrocellulose discs for processing by colony blotting. This involved lysing the colonies on the filter with 0.5N HC1 followed by removal of the unbound cell debris using a jet of water. The

protein binding capacity of the filter was then saturated using bovine serum albumin, after which the primary antibody was added.

This antibody had been raised in rabbits against whole live 569B bacteria and the rabbits had been boosted with a crude preparation of adhesin material. Unbound antibody was then washed away and the secondary antibody added. This antibody was a conjugate of goat-antirabbit antibody and horse radish peroxidase. After washing to remove unbound conjugate the enzyme substrate was added to visualize those colonies expressing Vibrio cholerae antigens which could react with the primary antibody. Positive colonies isolated in this way were purified, rechecked in a colony blot and the cloned DNA/vector isolated and used to retransform E. coli K12 to confirm that the positive reaction was a plasmid property.

Plasmid pPM440 (and pPM450 and pPM460)

The invention could reside in one of the plasmids pPM440, pPM450 and pPM460. These plasmids were cloned independently using Vibrio cholerae strain 017 as the source of donor DNA. The three plasmids are apparently identical and we have used pPM440 for further characterization. This plasmid contains an insert of about 2.2 kb and a restriction endonuclease cleavage map is shown in Figure 1. The term kb is used herein to DNA length and refers to the number of kilo-base pairs.

In order to identify the gene products which we had cloned and which were reacting with the antiserum in the colony blotting, cell envelopes were prepared. The envelopes were solubilized in SDS and run on an SDS-polyacrylamide gel. The material in the gel was then electrophoretically transferred to nitrocellulose which was in turn treated similarly to the procedure used for colony blotting. The result of such a transfer is shown in Figure 2. This clearly shows the presence of a band of about 22000 daltons present in pPM440 and not in either the vector or negatively reacting clones. This protein can also be seen in the cell envelopes after SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein staining.

Two deletion derivatives of pPM440 have been constructed. The first of these, pPM441, was made by deleting from the HindIII site in the vector DNA clockwise to the HindIII site in the cloned DNA (see Figure 1). The second deletion, pPM442, was obtained by deleting the DNA between the HpaI sites in the cloned DNA. Both of these deletions removed the ability to react with antiserum.

A series of transposon Tn1725 insertion derivatives of pPM440 have been isolated: pPM443 - pPM448. Of these, strains harbouring pPM447 and pPM448 both give a positive colony blot although pPM447 is weaker than pPM448 and the parent plasmid pPM440. The other plasmids do not react at all.

If one examines by SDS-PAGE the cell envelopes of strains harbouring these plasmids, both deletion

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and insertion, then pPM448 produces normal levels of the 22000 dalton protein, compared to pPM440, whereas pPM447 has reduced levels and all the others have no detectable protein (Figure 3).

In Figure 4 a composite of all the restriction analyses of these various plasmids is shown. Using these data and the observation that the Tn1725 insertion in pPM447 is probably a polar insertion leading to lowered levels of the 22000 dalton protein, the position of the DNA encoding the 22000 dalton protein can be mapped as shown at the bottom of Figure 3. The NH_2-terminus of the protein is probably encoded at the right hand end.

If one examines the proteins encoded by all of these plasmids in minicells then the same result is obtained (Figure 5). These results also show that the only new protein detected in pPM440 as compared to the vector, pBR322, is the 22000 dalton protein and that this protein is coded for on the plasmid.

Southern blotting analysis

Using the technique of DNA/DNA hybridization according to Southern we have used pPM440 as a probe to look at the similarity of this region of the chromosome in different Vibrio cholerae strains. Analyses using three different restriction enzymes demonstrated that the DNA cloned in pPM440 is present in both Classical and E1 Tor strains, and no discernible difference could be detected between strains. This implies that the gene and protein are common to both Biotypes of Vibrio cholerae.

Characterization of the pPM440 protein

The 22000 dalton protein encoded by pPM440 has been partially characterized and purified. In Figure 6 are shown the results of SDS-PAGE after fractionating the cell envelope of cells of E. coli K12 harbouring the plasmid pPM440. The left panel shows the results obtained by staining the gel and the right panel shows a duplicate gel which has been analysed by a Western blot using Vibrio cholerae antiserum to identify the pPM440 protein. The results of the fractionation imply that the pPM440 protein is probably a peptidoglycan-associated outer membrane protein.

Fractionation of the protein in this way followed by ion-exchange chromatography on hydroxyapaptite in SDS followed by gel filtration on Biogel P150 in SDS has enabled the protein to be purified.

Expression of the pPM440 protein in Salmonella

In order to carry out the relevant immunological testing and to determine whether or not the pPM440 protein is expressed in Salmonella, it was necessary to transform S. typhimurium strain G30 and S. typhi strain Ty21a with purified pPM440 plasmid DNA. The plasmid vector was also introduced as a control. Cell envelopes of these strains, Vibrio cholerae 569B and the original E. coli isolates were prepared and analysed by SDS-PAGE. The results are shown in Figure 7. It can be seen that the pPM440 protein is only a minor protein in Vibrio cholerae,, not being readily detected. However, in the cloned form in E. coli, S. typhimurium and S. typhi the protein is a major band.

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All of these strains have been analysed for their ability to be agglutinated by Vibrio cholerae 569B antiserum. The results of these agglutinations are shown in Table 3. In these tests it was observed that when pPM440, and thus the 22000 dalton protein, was present the cells showed significant autoagglutination. This is suggestive of the 22000 dalton protein being an adherence factor. The data show that antibodies to vibrio cholerae can agglutinate the strains harbouring pPM440. Thus not only is the protein being expressed in the Salmonella vaccine strains, but it is being expressed in a form which is recognizable by antibodies.

pPM440, therefore, appears to be useful in the form of an oral vaccine as described by this invention.

The invention could thus reside in immunizing an animal recipient (including man) by the steps of introducing into the animal by oral administration a pharmaceutically or physiologically acceptable carrier carrying therewith a Salmonella typhi galactose-epimeraseless (galE) mutant expressing the Vibrio cholerae surface antigen which we have cloned in the plasmid pPM440.

The invention could reside in the surface antigen being encoded on a plasmid.

The invention could reside in the surface antigen being chromosomally encoded, having been genetically engineered into the Salmonella typhi chromosome.

TABLE 3

AGGLUTINATION BY
ANTISERUM TO LIVE VIBRIO CHOLERAE 569B

Strain		Titre +
V. cholerae 569B		1000
E. coli K12		< 50
	+pBR322	< 50
	+pPM440	500*
S. typhimurium G30	+pBR322	< 50
"	+pPM440	500*
S. typhi Ty21A	+pBR332	< 50
	+pPM440	1000*

+Titre is the reciprocal of the lowest dilution giving agglutination.

*Significant Autoagglutination

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Plasmid pPM500

A further example of this type could be the protein encoded by the plasmid pPM500. This protein is about 16-18 kDaltons in size and reacts very readily with antisera in a Western blot (Figure 8).

Plasmid pPM500 consists of a 6.7 kb Pst1 fragment of V. cholerae DNA cloned into the Pst1 site of pBR322. The gene for the pPM500 protein has been localized by transposon mutagenesis and corresponds to a region of 400-500 base pairs spanning the HindIII site at 10.4 kb on the pPM500 map (Figure 9).

Minicell analysis to detect the plasmid encoded proteins suggest that the pPM500 protein lacks methionine.

Plasmid pPM510 (and pPM520)

A further example of this type could be the plasmid pPM510 (Figure 10) which encodes two proteins. These proteins are about 22 kDal and 60-65 kDal in size and both react strongly in a Western blot (Figure 8).

Plasmid pPM510 consists of a 5.1 kb Pst1 fragment cloned into the Pst1 site of pBR322. The location of the genes for the proteins within the cloned DNA has not yet been determined but together they would take up about 50% of the coding capacity of the cloned fragment.

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of V.cholerae (Figure 11), encoded on each of one of the plasmids pPM451, pPM455, pPM472 and pPM530. Detailed analysis has been performed using pPM455 and a restriction map of this plasmid is shown in Figure 12. The 25 kDal protein has been identified by Western blot analysis (Figure 13) and the location of the gene within the cloned DNA has been determined by deletion and transposon insertion analyses (Figure 14). These analyses show that the OmpV gene lies between the point of insertion of Tn1725 in plasmid pPM551 and the HindIII site at 7.0 kb. Other studies involving generating fusion proteins in part derived from OmpV show that transcription is from left to right.

The OmpV protein appears to be very immunogenic (Figure 15) and besides being a major outer membrane protein it is part of the sheath of the flagellum. Thus antibodies to this protein should immobilize the bacterium by binding to the flagellum, and in so doing prevent the V. cholerae from colonizing the gut.

Plasmid pPM431

A further example of this type which could be used in combination with others to increase protection is encoded by plasmid pPM431 (Figure 16). This plasmid encodes several of the proteins associated with haemolysin production (Figure 17). The genes have been localized by transposon insertion analysis and by determining

the plasmid encoded proteins in minicells (Figure 18). These results have enabled a partial physical map of the plasmid to be determined (Figure 19). Most importantly, HlyA the structural gene for the haemolysin is encoded within this DNA.

The importance of the haemolysin protein in protection is that it has been shown to be diarrhoeagenic in rabbit ileal loops. Thus, antibodies in the gut could be expected to be beneficial in reducing diarrhoea due to this excreted protein.

Deletion of the gene(s) determining haemolysin production or part thereof in attenuated V. cholerae strains may also be useful in generating vaccine strains different to the type described in this document. In particular, if haemolysin is responsible for some diarrhoea in cholera-toxin-less V. cholerae then deletion or inactivation would greatly improve those strains.

EXAMPLE 5

Very specific difficulties were experienced in relation to this example.

It was suspected that an O-antigen of Vibrio cholerae could be useful.

It was known that an antibody directed against Vibrio cholerae O-antigen could be very effective in protection against Vibrio cholerae in animal model systems and in man. It was also known that the O-antigen induces a good immune response and hence would be the ideal antigen for a vaccine strain of the above type.

It was known from work with respect to Salmonella that the genes which enable the synthesis of the O-antigen polysaccharide monomer are mostly present in one cluster of genes, located at about 42 minutes on the Salmonella genetic map. Some of these genes encode enzymes for synthesis of constituent monosaccharides and others encode enzymes for assembly of the monomer on a lipid carrier. It was guessed at that this gene cluster would occupy a substantial segment of DNA.

It was also known from work with respect to Salmonella that the genes which determine polymerisation of the O-antigen monomer to O-antigen polymer and the genes which encode the translocase which translocates the O-antigen polymer to the lipopolysaccharide (LPS) core to give the complete lipopolysaccharide molecule are both located away from the major gene cluster.

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It was to be expected by comparison with Salmonella that the genetic basis for Vibrio cholerae O-antigen synthesis would be equally complex and further that even if the entire set of genes necessary for synthesis of Vibrio cholerae O-antigen were cloned and transferred to E. coli or Salmonella, that the O-antigen polymer may not be transferred to the E. coli or Salmonella lipopolysaccharide core which differs from the Vibrio cholerae LPS core.

The difficulties in cloning the O-antigen synthesis system such that it can be expressed on the LPS of E. coli or Salmonella are thus firstly that the major gene cluster may occupy a substantial length of DNA, secondly that there may be other genes essential for full expression not present in the main cluster and thirdly, that the genes even when cloned and placed together in E. coli or Salmonella may not fully express the O-antigen on the cell surface due to differences in the lipopolysaccharide cores of these organisms and Vibrio cholerae, such that the translocases would not transfer the Vibrio cholerae O-antigen polymer to the E. coli or Salmonella core.

Because of the anticipated enormous difficulties in cloning the full set of genes necessary for Vibrio cholerae O-antigen to be expressed on the surface of E. coli or Salmonella, we have been looking primarily for clones encoding surface proteins of Vibrio cholerae which may induce effective immunity when present in suitable vaccine strains of Salmonella typhi or a related organism. However, in addition, we conducted our experiments in such a way that should a set of genes be cloned which enabled the O-antigen of Vibrio cholerae to be expressed on the surface of E. coli, then that would be detected by our screening method.

In our first attempts at cloning, we used the cosmid vector pHC79 as such a vector should enable the cloning of clusters of genes on DNA of the order of 30-40 kb in size. However, the Vibrio cholerae DNA obtained by the conventional methods was of low molecular weight, presumably due to partial degradation by the extracellular DNAase produced by Vibrio cholerae. We therefore had to proceed with cloning into plasmid vectors and obtained many clones which expressed Vibrio cholerae surface proteins in E. coli, but all contained only small pieces of Vibrio cholerae DNA, averaging from 2 kb to 8 kb depending on the restriction enzyme used. We then devised a method for extracting Vibrio cholerae DNA in the presence of pronase, such that it remained in a high molecular weight form which could be then cut by restriction enzymes into pieces of 30 to 40 kb in size suitable for cloning into cosmids.

We have now discovered that indeed Vibrio cholerae carries genes which are within a common locality such that they can be cloned as a group into a cosmid and determine the synthesis of the Vibrio cholerae O-antigen in E. coli.

Furthermore, it has been discovered that the DNA length effective for this purpose is large but less than 40 kb.

Furthermore, we have discovered that there is indeed a technique which when used with respect to such material as Vibrio cholerae chromosomal DNA can keep this material in sufficiently high molecular weight pieces while at the same time providing sufficient concentration and purity of such material that this can be useful to obtain fragments in the 40 kb pair range and which are useful for subsequent cloning techniques.

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We have discovered that if the cells are lysed in the presence of pronase and if separation and concentration is effected using solvent extraction where the chromosomal DNA is kept within the same container during this process, then the DNA strands can be isolated with very long lengths.

The integrity of the DNA is also assisted by the fact that the extraction process can be effected somewhat quicker than if centrifugation on Caesium chloride gradients is used and this reduces the time during which DNAase is kept in contact with the otherwise integral material.

The specific preferred technique for obtaining such extended lengths of DNA or further cloning techniques will be described in a later part of this specification.

Specifically however, having been able to obtain chromosomal DNA of large molecular weights from each of Vibrio cholerae strains 569B (a classical Inaba strain) and 017 (El Tor, Ogawa), this chromosomal DNA was then partially digested by using an appropriate restriction endonuclease which could be expected to leave at least some fragments in the range of approximately 35 through 50 kb pairs.

In preference, the restriction endonuclease Sau3A was used for this purpose and was indeed effective to provide appropriate restriction fragments.

Proceeding on the assumption that even a protein antigen suitable for vaccine purposes might be better expressed from a gene cluster capable of being contained within a DNA strand of approximately 40 kb pairs in length cloning into a cosmid vector was proceeded with. Accordingly DNA of the cosmid vector, pHC79, was completely digested with BamHI and subsequently treated with alkaline phosphatase to prevent self ligation.

The restriction fragments from the Vibrio cholerae and the cosmid vector DNA were then mixed, ligated overnight, and packaged in vitro into bacteriophage λ. The phage lysate was then used to infect a restrictionless (hsdR), recombination deficient (recA) Escherichia coli K-12 strain. Cosmid containing cells were selected on nutrient media containing ampicillin. Greater than 90% of these colonies obtained comprised cells which were sensitive to tetracycline (as expected if DNA is cloned into the BamHI site of the vector) which could be assumed to carry cosmid pHC79 with cloned Vibrio cholerae DNA. Such cells contained thereby cosmid clones containing between 35 to 50 kb pairs of DNA.

The colonies were then tested with antibody to determine if any produced Vibrio cholerae antigens as a result of carrying cloned Vibrio cholerae DNA.

Accordingly, the cosmid clones as obtained were then patched onto numbered grids on nutrient medium containing ampicillin and grown up overnight at 37°C. They were then transferred to nitrocellulose discs for processing by colony blotting. This involved lysing the colonies on the filter with 0.5N HC1 followed by removal of the unbound cell debris using a jet of water.The protein binding capacity of the filter was then saturated using skim milk powder after which the primary antibody was added.

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This antiserum or antibody had been raised in rabbits against whole live 569B bacteria and the rabbits had been boosted with a crude preparation of adhesin material. Unbound antibody was then washed away and the secondary antibody added. This antibody was a conjugate of goat-anti-rabbit antibody and horse radish peroxidase. After washing to remove unbound conjugate the enzyme substrate was added to visualize those colonies expressing Vibrio cholerae antigens which could react with the primary antibody. Positive colonies isolated in this way were purified, rechecked in a colony blot and the cosmid DNA isolated and used to retransform E. coli K-12 to confirm that the positive reaction was associated with the cosmid DNA. The positively reacting colonies were then further analysed by repeating the colony blotting and using other antisera in place of the anti-live-569B serum, including the anti-live-569B serum described above after it had been extensively adsorbed with purified LPS from Vibrio cholerae 569B to remove antibodies reacting with the LPS.

Clones encoding genes for the synthesis of the LPS should be detected by their inability to react in a colony blot with this antiserum after it has been extensively adsorbed with purified LPS.

According to this method several clones were identified as containing genes effective for the synthesis of the O-antigen of the LPS.

Our invention accordingly could reside in restriction fragments of DNA containing less than 40 kb of DNA and containing in a common location such genes effective for the determintion of the O-antigen.

The invention then could be said to reside in the combination of the genes effective for the synthesis of O-antigen with a cosmid vector to produce thereby a cosmid clone this being a clone of the size between 35 to 50 kb pairs of DNA.

pPM1001 is exemplary and accordingly our invention can reside in the cosmid pPM1001.

Subsequent analysis of this cosmid reveals that it contains approximately 36 kb pairs Vibrio cholerae DNA cloned into pHC79. A gel electropherogram of the said DNA restricted with several endonucleases is shown in FIG. 23.

The presence of this cosmid identified as pPM1001 in the bacterial cell renders it capable of producing the O-antigen of the LPS of the Inaba serotype.

This material can be detected in several ways.

If cell envelopes are isolated from the bacteria harbouring pPM1001, solubilized in SDS and analysed by polyacrylamide gel electrophoresis followed by staining with silver then it is possible to detect the production of a ladder of bands typical of lipopoly-saccharide containing O-antigen (FIG. 24). This same envelope material can also be electrophoresed in the same way, transferred electrophoretically to nitro-cellulose and Western blotted using antiserum to the

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LPS. In this way, it is possible to detect the O-antigen material, by first reacting the nitrocellulose sheet with rabbit anti-Vibrio cholerae LPS serum, followed by goat-anti-rabbit serum to which has been coupled horse radish peroxidase and the immune complexes can be visualized by adding substrate for the peroxidase. Only the V. cholerae O-antigen is visualized in such a Western blot, and the positive reaction obtained showed that the LPS carried Vibrio cholerae O-antigen.

Purified LPS has also been extracted by the hot phenol-water method from E. coli K-12 harbouring either pHC79 or pPM1001. Upon electrophoresis followed by silver staining the presence of pPM1001 can be seen to lead to the ladder of bands typical of LPS carrying O-antigen (FIG. 25).

The purified LPS has also been used in a haemagglutination inhibition assay to see if it is capable of neutralizing anti-LPS immunoglobulin so as to prevent the agglutination of sheep red blood cells which have Inaba LPS fixed upon their surface. This assay demonstrated that the O-antigen material produced in E. coli K-12 as a result of harbouring pPM1001, was immunologically indistinguishable from the LPS of the Inaba parent V. cholerae strain 569B. LPS from the clone, Ogawa LPS or Inaba LPS, each in 2 fold serial dilutions, was used to inhibit either the agglutination by anti Ogawa serum of sheep red blood cells (rbc's) coated with Ogawa LPS or the agglutination by anti-Inaba serum of sheep rbc's coated with Inaba LPS.

In the Inaba coated rbc experiment, LPS from the clone inhibited to well 11 (1 ug/ml) compared to well 12 (.5 ug/ml) for V. cholerae Inaba LPS. Ogawa LPS inhibited to well 7 (16 ug/ml). Thus LPS from the clone inhibits very well showing that it has all or most of the specificity of Inaba LPS.

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In the Ogawa coated rbc experiment LPS from all three types inhibited to about well 15 (.06 ug/ml). Thus the LPS from the clone inhibits to the same extent as V. cholerae Inaba LPS, showing that it carries the common antigenic specificities known to be shared by LPS from Inaba and Ogawa V. cholerae strains. LPS from E. coli carrying pHC79 gave virtually no inhibition in either system.

As already mentioned this LPS carrying O-antigen material being produced as a result of the cells harbouring pPM1001 is found in the cell envelope. It can further be shown to be exposed upon the cell surface since cells harbouring pPM1001 can be agglutinated by anti-LPS serum whereas cells harbouring no cosmid or corryin pHC79 cannot.

pPM1001 encodes the genes determining the Inaba serotype of V. cholerae. However, variation between the serotypes (Inaba and Ogawa) of V. cholerae has been localized to one locus and now that we have found that all the genes necessary forthe expression in E. coli of Inaba O-antigen specificity can be cloned on one DNA fragment, it seems that through the invention the genes necessary for the expression in E. coli of Ogawa O-antigen can also nbe cloned on a single DNA fragment.

pPM1001 can be used to obtain a cosmid clone from a gene bank constructed using DNA from a vibrio cholerae (Ogawa) strain, such as 017: this could be done simply by using pPM1001 or part of the cloned vibrio cholerae DNA encoding O-antigen synthesis as a DNA probe to detect the Ogawa clone. Alternatively, the Ogawa genes could be cloned by the same procedure as used to isolate pPM1001 (except that an Ogawa antiserum must be used. In fact, we have succeeded in the isolation of the cloned Ogawa antigen by the same procedure. Plasmids pPM1002 and pPM1003 are exemplary and accordingly our invention could reside in pPM1002 and pPM1003.

Thus, pPM1001, pPM1002 and pPM1003 appear to be useful in the formulation of a vaccine as described by this invention.

The invention could be said to reside in the oral ingestion by man of a pharmaceutically and physiologically acceptable carrier expressing the vibrio cholerae O-antigen of the lipopolysaccharide.

In a further form, the invention could reside in the replication of bacterial strains carrying therewith a plasmid carrying the genes encoding enzymes which synthesise O-antigen.

In a further form, the invention could be said to reside in a bacterial strain providing for the expression of O-antigen of Inaba type which also has antigenic specificity shared with Ogawa type O-antigen.

In a further form, the invention could reside in a bacterial strain useful as an oral vaccine to assist in promotion of immunity in man to Vibrio cholerae where such bacterial strain is of a type which will invade the lymphoid tissue of the small intestine for a limited but sufficient period of time, and being a strain rendered non pathogenic such that the invasive characteristic relevant to the lymphoid tissue of the small intestine remains, the derived strain retaining the above characteristics also carrying therein genes enabling the synthesis of the O-antigen of the Vibrio cholerae.

The invention could reside in the method of promoting immunisation to Vibrio cholerae of man which comprises the oral ingestion of the said bacterial strain.

It is implicit throughout this description that the effective value of the O-antigen is such to promote immunisation or at least significant resistance to the pathogen Vibrio cholerae.

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To assist in a further understanding of this invention there is further attached hereto analysis of materials thus far produced as set out herein in this last example.

The invention could reside in the method of preparing in the first instance appropriate DNA in which the steps of separation and concentration do not include centrifugation or extraction using pipetting or the like and includes pronase to prevent enzyme degradation of the DNA.

We have developed a procedure which allows us to rapidly isolate Vibrio cholerae or Salmonella typhimurium or other bacterial DNA suitable for recombinant DNA use, without recourse to CsC1 centrifugation or long incubation times.

METHOD: Vibrio cholerae strains 569B (Classical, Inaba) and 017 (El Tor, Ogawa), and Salmonella typhimurium LT 2 have been successfully used for purification of chromosomal DNA. The procedure is described below.

Cells were grown overnight with aeration at 37° in 20 ml of Brain-Heart infusion broth (DIFCO). The cells were then centrifuged at 5000xg for 10 min at room temperasture and washed once in TES buffer (50mM TrisHC1, 5MM EDTA and 50mM NaC1 pH 8.0), followed by centrifugation again, and the washed cells were then resuspended in one-tenth of the original volume (i.e., 2.0 ml) in cold 25% sucrose in 50mM Tris-HC1 pH 8.0. To this was added 1.0 ml of 10 mg/ml lysozyme in 0.25M EDTA pH8.0, and the suspension was allowed to stand on ice for 20 min. TE bufer (10mM Tris-HC1, 1mm EDTA pH 8.0) (0.75 ml) was then added followed

by 0.25 ml of lysis solution (5% sarkosyl, in 50mM Tris-HC1, 0.25M EDTA pH 8.0). Solid pronase (10 mg) was also added to the mixture and gently mixed. This was incubated for 60 min at 56° with occasional shaking. Lysis becomes apparent at this time.

The lysate was then transferred to a 50 ml Ehrlenmeyer flask and gently extracted three times with phenol saturated with TE buffer (10 mm Tris-HCL, 1mMEDTA pH8.0). This is usually made by adding 1 g of phenol per ml of TE. The residual phenol is then removed by extracting twice with diethyl-ether. Two volumes (8 ml) of cold (15°C) 95% ethanol were then added to the clear viscous DNA solution.

This resulted in the precipitation of a large mass of stringy DNA. This mass readily settles and the aqueous ethanol solution can be decanted since the DNA also tends to adhere to the glass. The DNA was then washed twice with 70% ethanol in a similar manner.

The washed DNA was then dried under vacuum for approximately 60 min, after which 1.0 ml of TE buffer was added and heated at 56°C for a few minutes. The DNA solution was then transferred to a 1.5 ml Eppendorf tube and any residual particulate material removed by centrifugation for 2 min in an Eppendorf 5414 table top centrifuge. The supernatant was transferred to a fresh tube and stored at 4°C. The concentration of DNA was usually in the range of 200-500 ug/ml.

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RESULTS AND DISCUSSION: FIG. 26 shows the results obtained by restriction endonuclease digestion followed by 3 hour ligation of either V. cholerae or S. typhimurium DNA. It can be seen that the DNA could be fully cut with either EcoR1 or BamH1 and that upon ligation it moves back to high molecular weight material such that significant amounts remain trapped in the sample well.

The material shown has been pipetted using normal disposable tips (Gilson C20), however, if the aperture is increased by slicing off the tip with a scalpel blade the apparent molecular weight of the uncut and ligated DNA is significantly higher. It should be noted that no pipetting of the DNA is at all involved in the procedure and all manipulations are such as to minimize shearing.

The method has several advantages over the widely used method of Marmur in that only small volumes of organisms are required (20 ml) and that there are no extended or overnight incubations, indeed the whole procedure can be completed in three hours or less, yielding sufficient DNA for many cloning experiments. More recent methods involve overnight CsCl centrifugation which is time consuming and appears not to be necessary to obtain DNA of sufficient quality for molecular cloning experiments.

In addition to the use of DNA prepared by this procedure for cloning O-antigen genes, this procedure has been used in this laboratory for successfully cloning the Vibrio cholerae genes for several outer membrane proteins and for haemolysin production.

The method has also been used for the construction of several Vibrio cholerae cosmid banks, which necessitated having restriction fragments of greater than 38 kb. There is no reason why this method should not prove successful with other gram negative organisms and has also been used successfully in our laboratory for making a cosmid bank of Salmonella typhimurium.

1. A bacterial strain characterised by having a gene set substantially derived from a non pathogen or a pathogen rendered non pathogenic and able to invade, multiply, and briefly colonize the lymphoid tissue of the small intestine of mammalian species and having an introduced gene, genes or part thereof effective to specify synthesis of an antigen or antigens effective to generate a protective immune response due to production of IgA antibodies into the small intestine and other secretory surfaces.

2. A bacterial strain as in Claim 1 wherein the IgA antibodies produced are those effective against any one of the following genera where this is a pathogen; Escherichia, Rotavirus, Vibrio, Aeromonas and Bordetella.

3. A bacterial strain as in Claim 1 wherein the introduced genetic material is within the chromosome of the strain.

4. A bacterial strain as in Claim 1 wherein the introduced genetic material is on a plasmid within the bacterial strain.

5. A bacterial strain as in any of the preceding claims wherein the non pathogenic bacterium is selected from one of the following genus; Escherichia, Salmonella or Shigella.

6. A bacterial strain as in any of the preceding claims wherein the introduced genetic material specifies the synthesis of any of the following; adhesins, outer membrane proteins or lipopolysaccharides of the pathogen.

7. A bacterial strain as in any one of the preceding claims wherein the introduced genetic material specifies the synthesis of the K88 antigen of an enterotoxigenic Escherichia coli.

8. A bacterial strain as in any one of the preceding claims 1-6 wherein the introduced genetic material specifies the synthesis of the K99 antigen of an enterotoxigenic Escherichia coli.

9. A bacterial strain as in any of the preceding claims 1-6 wherein the introduced genetic material specifies the synthesis of the colonization factor antigens of enterotoxigenic Escherichia coli pathogenic to man.

10. A bacterial strain as in the preceding claim wherein the introduced genetic material specifies the synthesis of one of the antigens CS1, CS2 or CS3 of a CFA/II producing enterotoxigenic Escherichia Coli.

11. A bacterial strain as in the preceding claim wherein the introduced genetic material resides in any of plasmids pPM440, pPM500, pPM510, pPM455 and pPM431.

12. A Vibrio strain in which all or part of the genetic material encoded within pPM431 has been deleted with the aim of making them less toxic or diarrhoeagenic.

13. A bacterial strain as in any one of preceding claims 1-6 wherein the introduced genetic material specifies the synthesis of a surface protein of Vibrio cholerae.

14. A bacterial strain as in Claim 1 wherein the introduced genes are effective to synthesise an O-Antigen of Vibrio cholerae.

15. A bacterial strain as in the last preceding claim in which the synthesized O-Antigen is present on the surface of Escherichia coli.

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16. A bacterial strain as in Claim 14 in which the synthesized O-Antigen is present on the surface of Salmonella.

17. A bacterial strain as in preceding Claim 14 in which the synthesized O-Antigen is present on the surface of Shigella.

18. A method of manufacture of a bacterial strain as characterised in any one of the preceding claims which includes the steps of introducing the selected genes through an appropriate vector into the non pathogenic carrier bacteria.

19. A fragment of DNA containing genes determinating the synthesis of an O-Antigen of Vibrio cholerae.

20. The combination of genes effective for the synthesis of an O-Antigen of Vibrio cholerae with a cosmid vector.

21. The combination of genes effective for the synthesis of the O-Antigen of the Inaba serotype of Vibrio cholerae with a cosmid vector pHC79, the whole comprising the cosmid pPM1001 or part thereof.

22. The combination of genes effective for the synthesis of the O-Antigen of the Ogawa serotype of Vibrio cholerae with a cosmid vector pHC79, the whole comprising the cosmid pPM1002, pPM1003 or part thereof.

23. The combination of a Vibrio DNA fragment selected from any of the following Cosmids pPM1001, pPM1002, and pPM1003 with any vector or introduced into the chromosome of the non pathogenic strain.

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24. The combination of genes effective for the synthesis of an O-Antigen of Vibrio cholerae with a cosmid vector, the cosmid vector being pHC79.

25. A vaccine useful for effecting immunity in mammalian species against enteric disease caused by Vibrio cholerae through oral ingestion by the species of the vaccine including a live bacterial strain as characterised in any one of the preceding claims 1-6 and 11-17.

26. A method of manufacture of a bacterial strain effective for the expression of one of the O-Antigen of Vibrio cholerae which includes the steps of separation and concentration of the chromosomal DNA of Vibrio cholerae using solvent extraction and concentration only while the chromosomal DNA is kept within the same container.

27. The cosmids pPM1001, pPM1002 and pPM1003 as identified in the specification.

28. The method of manufacture of a bacterial strain as in the preceding method claim further including the step of adding pronase to the chromosomal DNA.

29. The method of manufacture of a bacterial strain including the steps of extracting and concentrating fragments of DNA of Vibrio cholerae so as to maintain at least some of the fragments in the length of approximately 32 kb pairs, combining the fragments with an appropriate cosmid vector and then isolating any thus formed cosmid clone carrying genes arranged to enable the expression therefrom of one of the O-Antigens of Vibrio cholera.

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30. A vaccine useful for effecting immunity in mammalian species against enteric disease through oral ingestion by the species of the vaccine including a live bacterial strain as characterized in any one of the preceding claims 1-17.

31. The cloning of an O-Antigen gene cluster using a cosmid vector.

AMENDED CLAIMS

[received by the International Bureau on 01 July 1985 (01.07.85); original claims 11,12 and 13 amended; new claim 32 added; other claims unchanged (2 pages)]

8. A bacterial strain as in any one of the preceding claims 1-6 wherein the introduced genetic material specified the synthesis of the K99 antigen of an enterotoxigenic Escherichia coli.

9. A bacterial strain as in any of the preceding claims 1-6 wherein the introduced genetic material specifies the synthesis of the colonization factor antigens of enterotoxigenic Escherichia coli pathogenic to man.

10. A bacterial strain as in the preceding claim wherein the introduced genetic material specifies the synthesis of one of the antigens CS1, CS2 or CS3 of a CFA/II producing enterotoxigenic Escherichia Coli.

11. A bacterial strain as in any one of preceding claims 1-6 wherein the introduced genetic material specifies the synthesis of a surface protein of Vibrio cholerae.

12. A bacterial strain as in the immediately preceding claim wherein the introduced genetic material resides in any one of plasmids pPM440, pPM500, pPM510, pPM455 and pPM431.

13. A Vibrio strain in which all or part of the genetic material encoded within pPM431 has been deleted with the aim of making them less toxic or diarrhoeagenic.

14. A bacterial strain as in Claim 1 wherein the introduced genes are effective to synthesise an O-Antigen of Vibrio cholerae.

15. A bacterial strain as in the preceding claim in which the synthesized O-Antigen is present on the surface of Escherichia coli.

30. A vaccine useful for effecting immunity in mammalian species against enteric disease through oral ingestion by the species of the vaccine including a live bacterial strain as characterized in any one of the preceding claims 1-17.

31. The cloning of an O-Antigen gene cluster using a cosmid vector.

32. A bacterial strain as in claim 11 wherein the introduced genetic material resides in any one of plasmids pPM474, pPM476, pPM477, pPM483.

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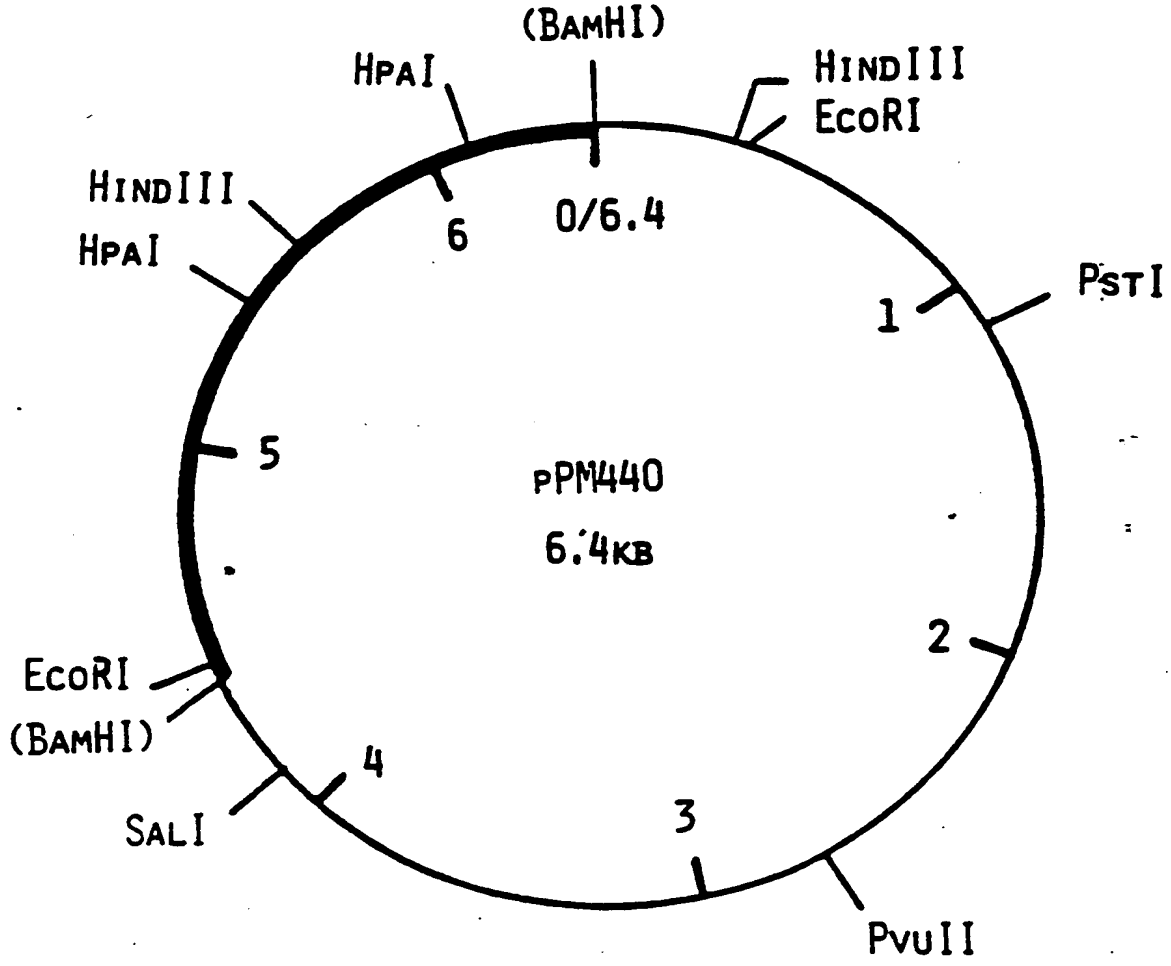


FIGURE 1:

Restriction map of pPM440 molecular sizes of the various fragments were calculated using bacteriophage SPP1 DNA digested with Eco R1 as a standard.

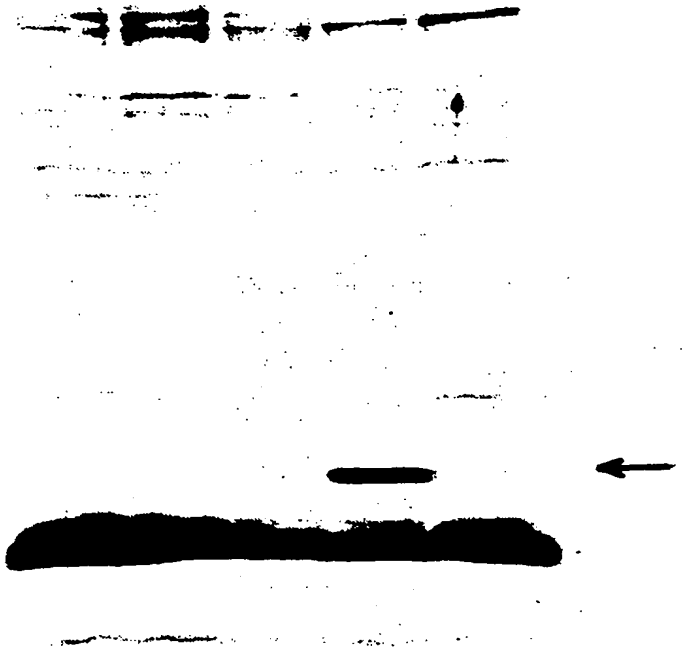


FIGURE 2:

Western blot analysis of cell envelopes of E. coli K-12 harbouring the various plasmids and using antisera against live Vibrio cholerae 569B.

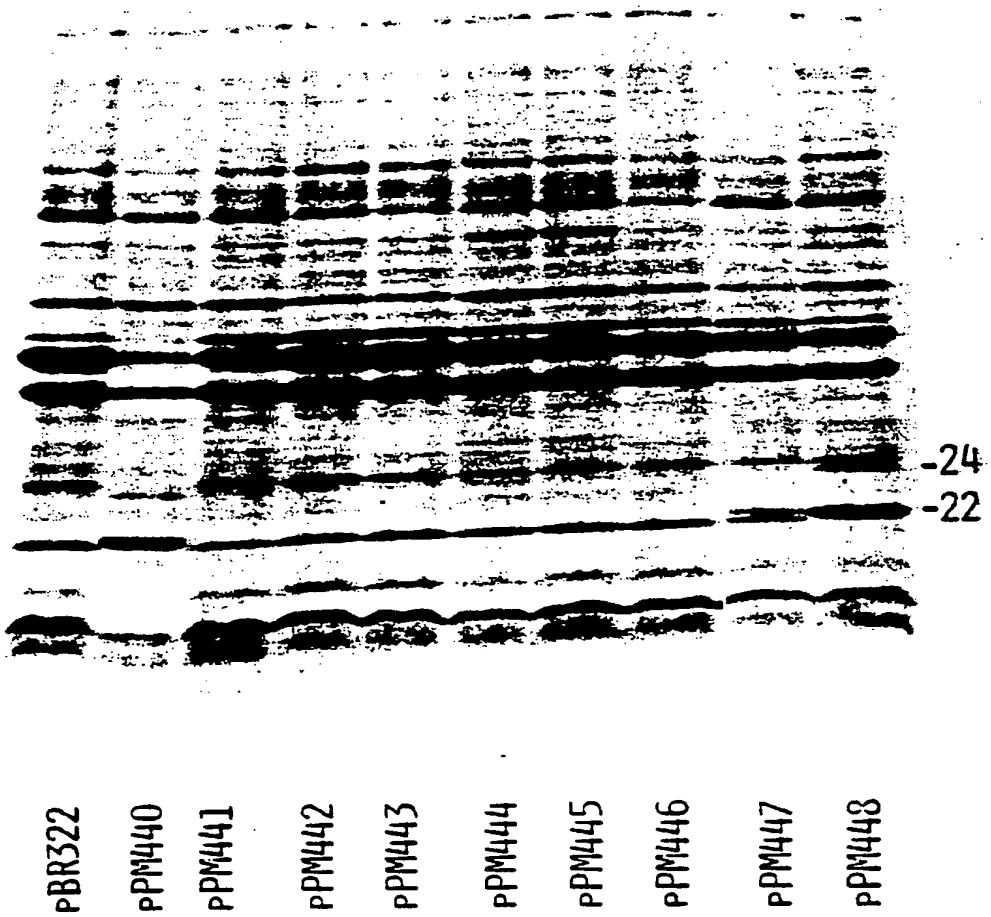


FIGURE 3:

Analysis by SDS-PAGE followed by staining, of cell envelopes of E. coli K-12 harbouring the various plasmids.

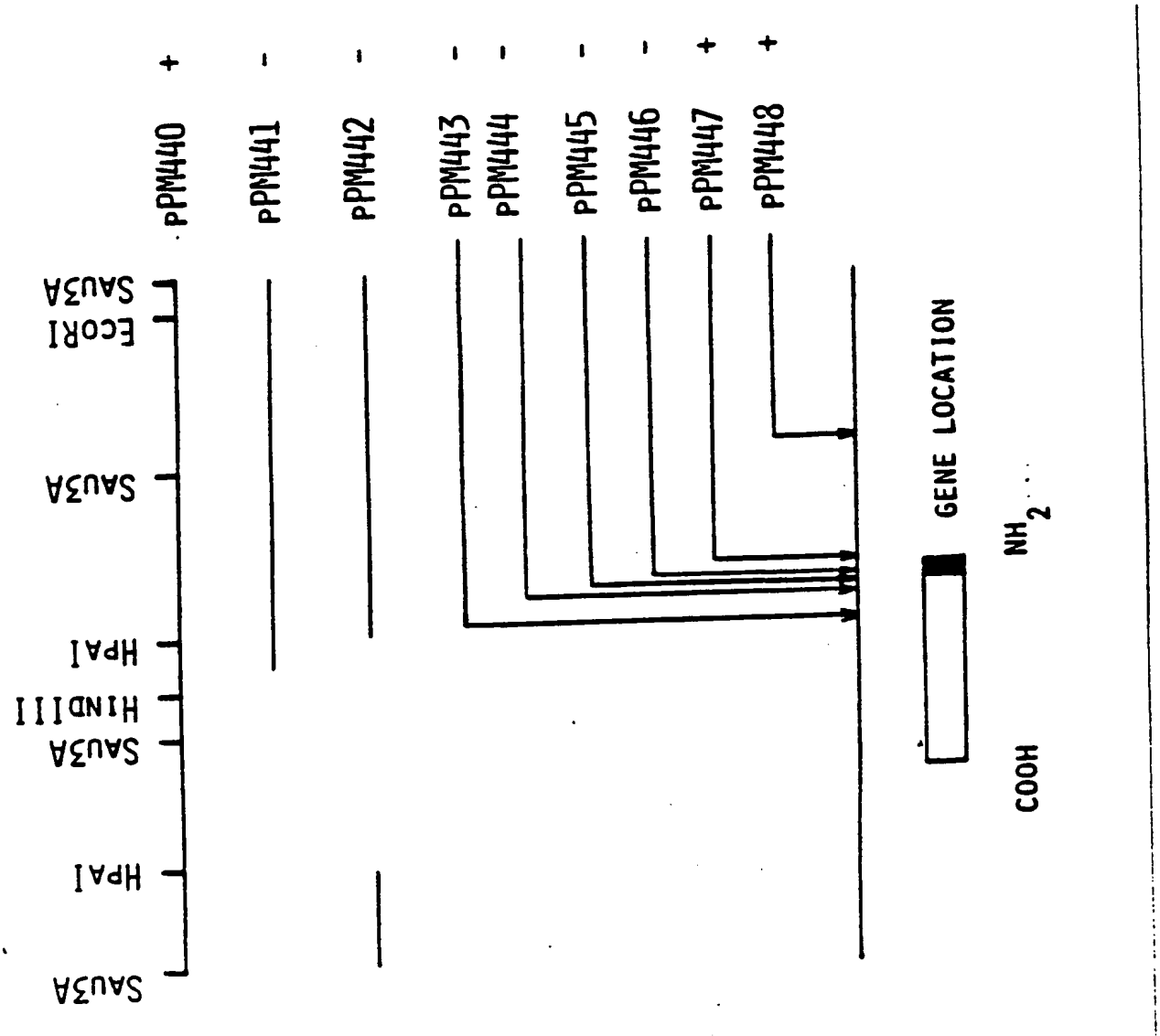


FIGURE 4:

Physical mapping of the gene for the 22000 dalton protein of pPM440 with respect to the deletions, pPM441 and pPM442, and transposon Tn1725 insertions, pPM443-pPM448.

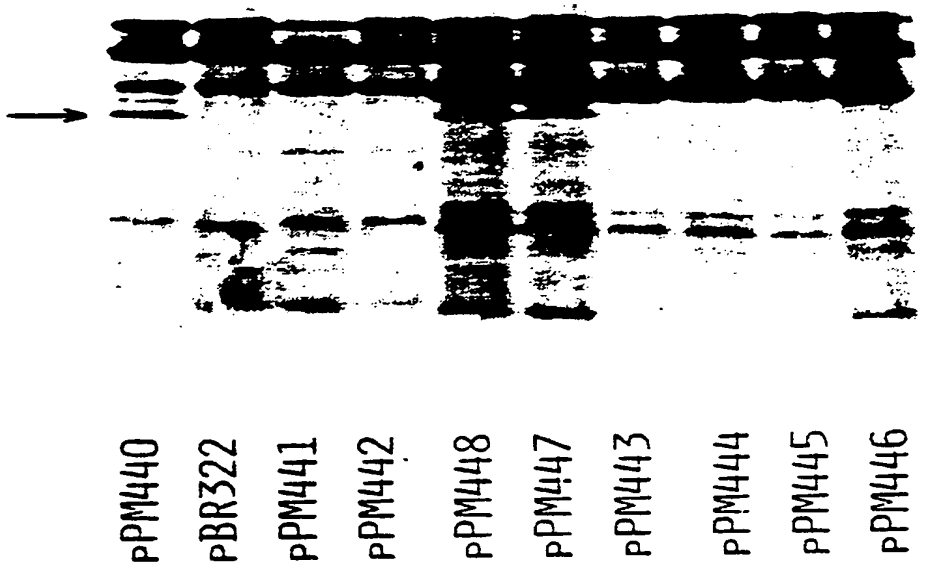


FIGURE 5:

Analysis of the plasmid encoded proteins in minicells harbouring the various plasmids by labelling with (^{35}S)-methionine, followed by SDS-PAGE and autoradiography.

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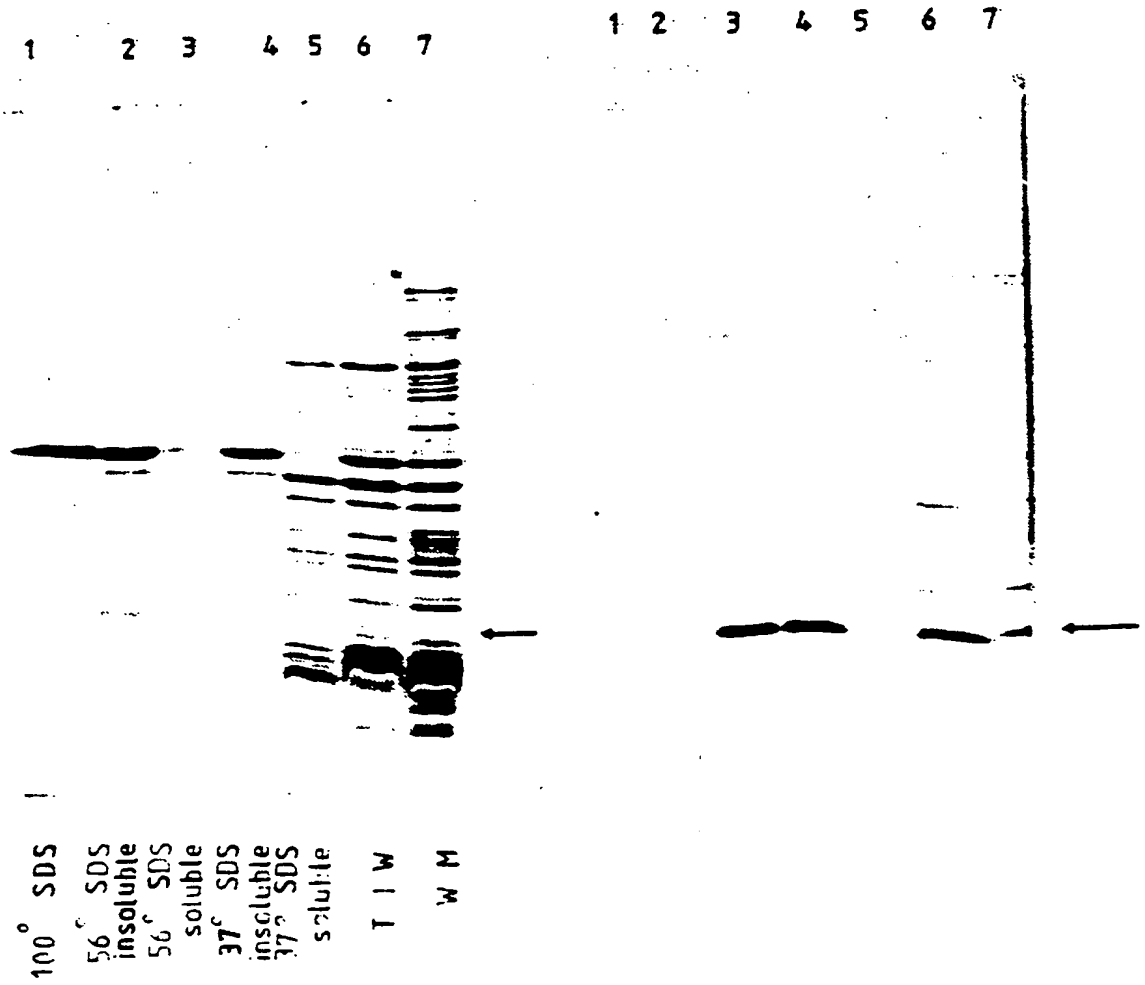


FIGURE 6:

Characterization of the 22000 dalton pPM440 protein by differential extractions following by SDS-PAGE and staining (left panel) or Western blot analysis with anti-live- V. cholerae 569B serum (right panel).

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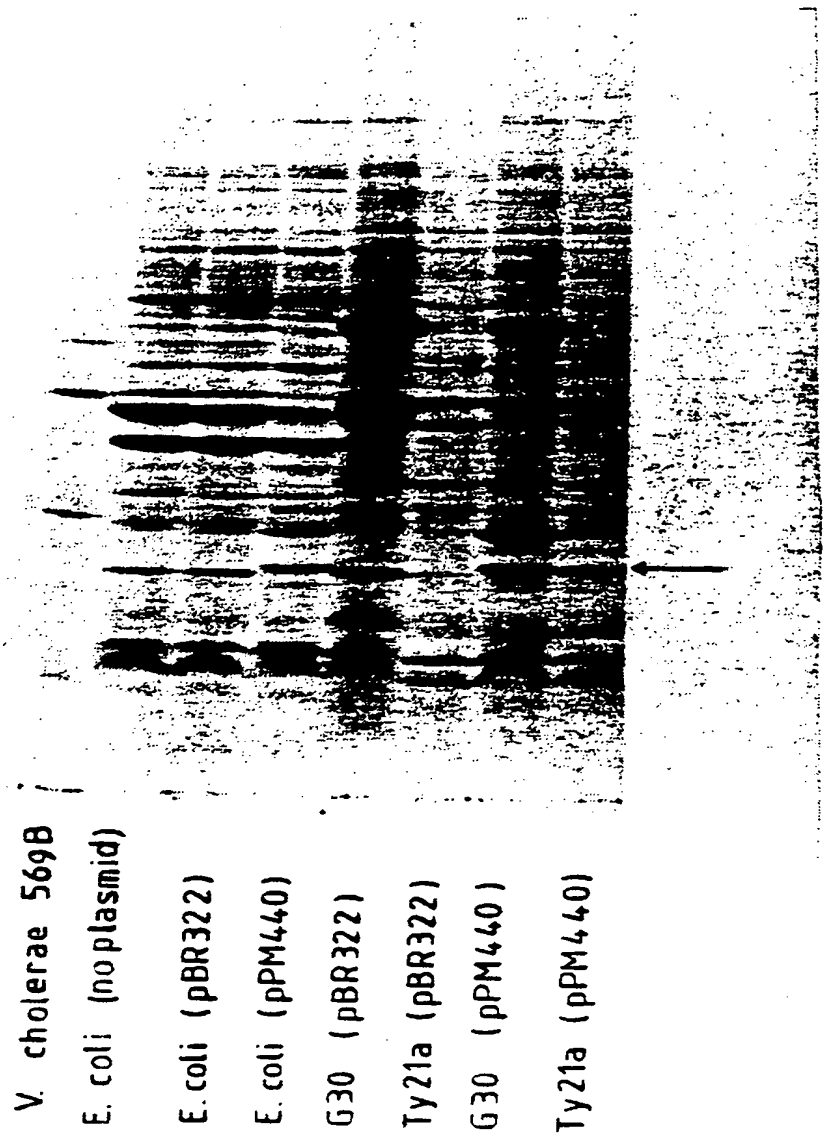


FIGURE 7:

Analysis by SDS-PAGE followed by staining of cell envelopes of the various strains harbouring the plasmids. The arrow indicates the cloned Vibrio cholerae surface antigen, the 22000 dalton protein.

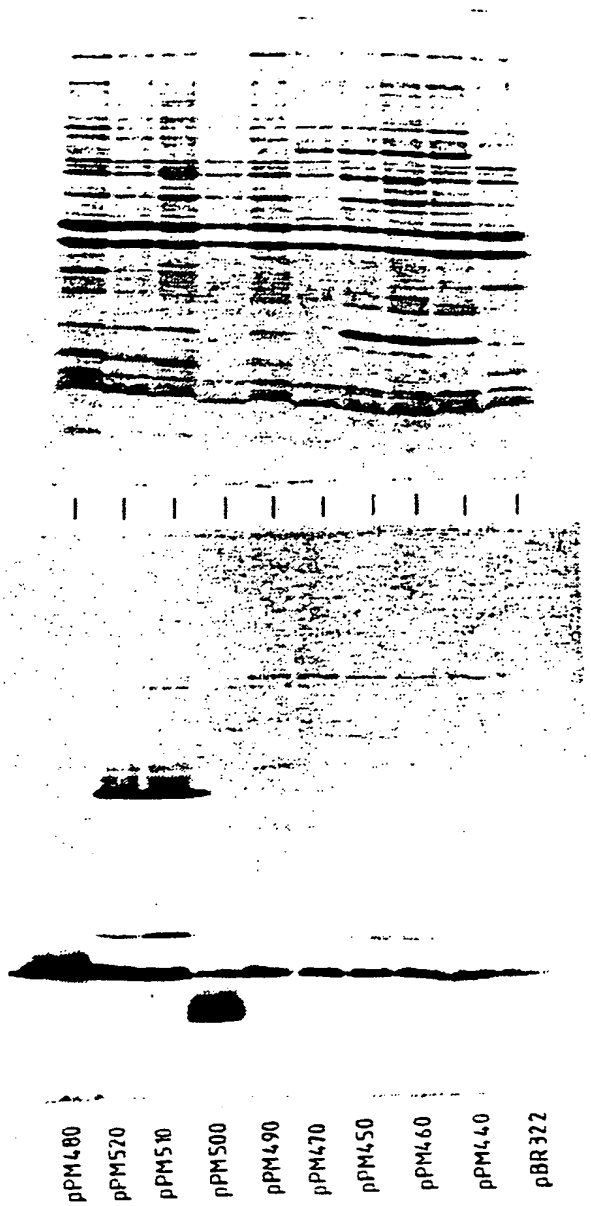


FIGURE 8:

Western blot analysis of E. coli K-12 derivatives harbouring the various plasmids. Cell envelopes of these strains were isolated and analysed on SDS polyacrylamide gels followed by either staining with Coomassie blue (upper panel) or by Western blot analysis using anti-live-569B serum after the proteins had been transferred to nitrocellulose (lower panel).

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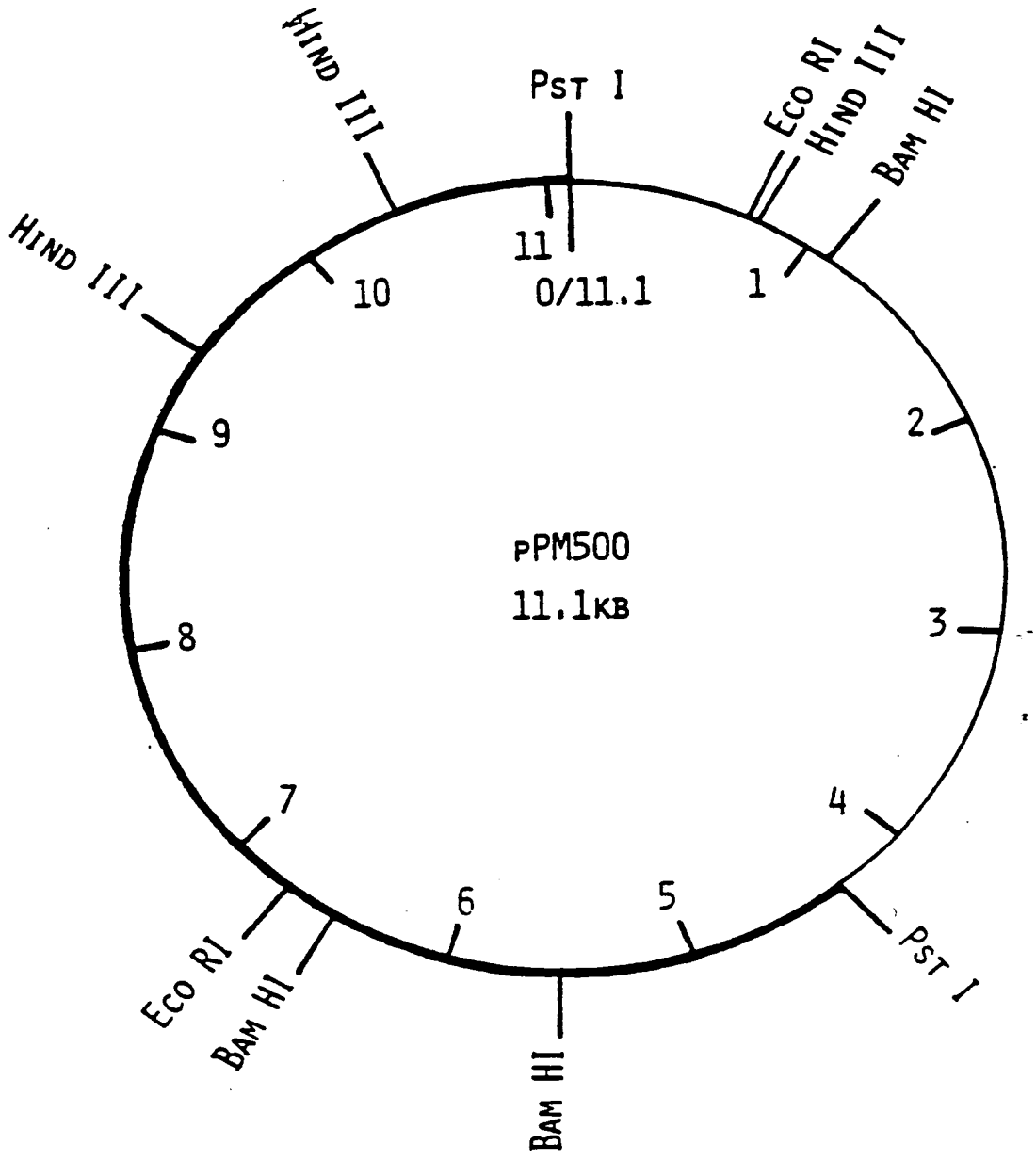


FIGURE 9:

Restriction map of plasmid pPM500. The thick line represents the region of cloned V. cholerae DNA.

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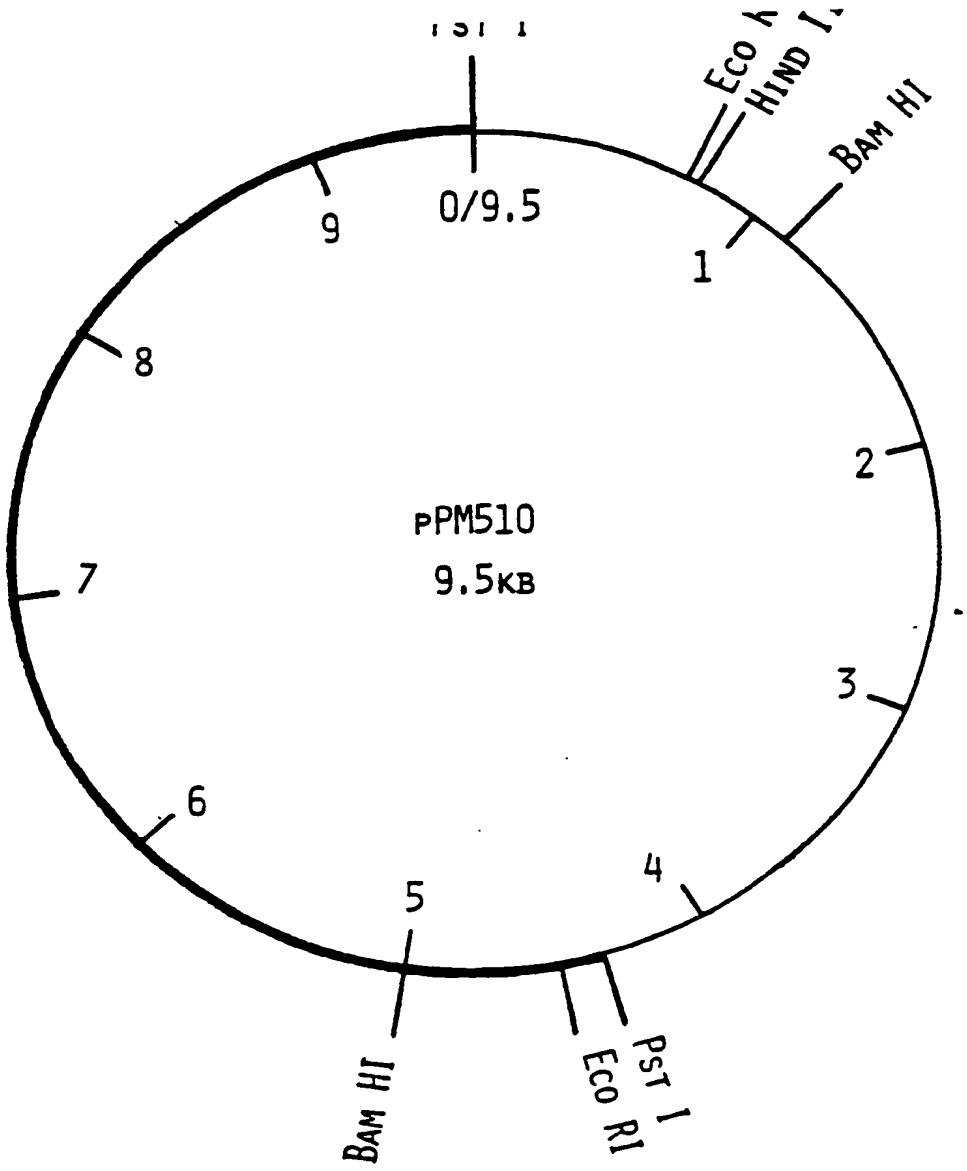


FIGURE 10:

Restriction map of plasmid pPM510. The thick line indicates the region of cloned V. cholerae DNA.

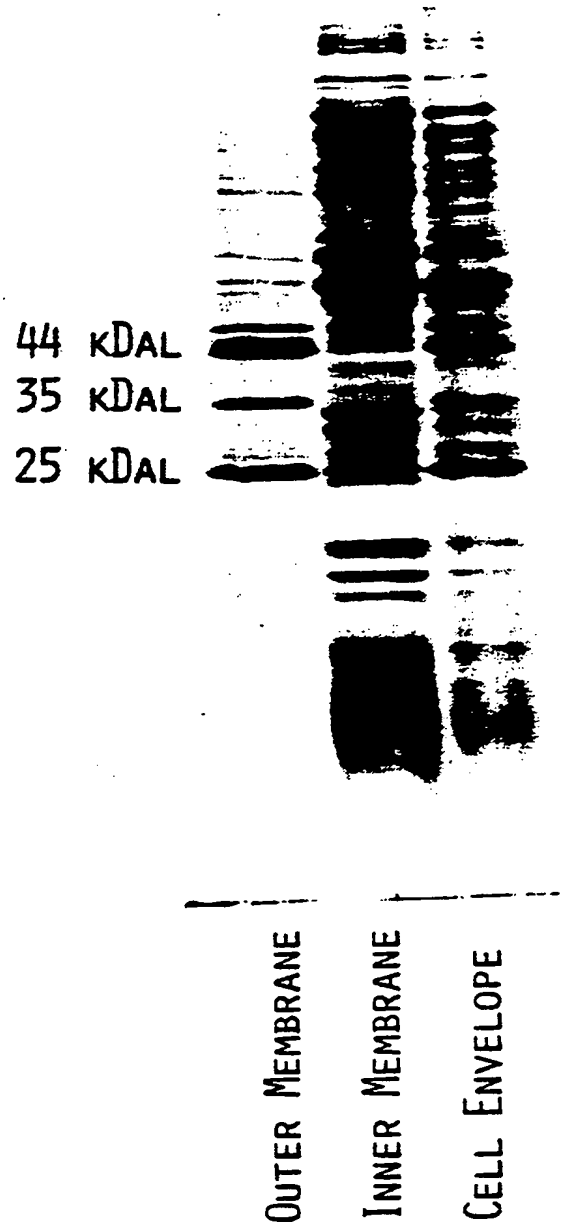


FIGURE 11:

SDS polyacrylamide gel electrophonesis of whole cell envelopes of V. cholerae and inner and outer membranes obtained by separation on sucrose density gradients.

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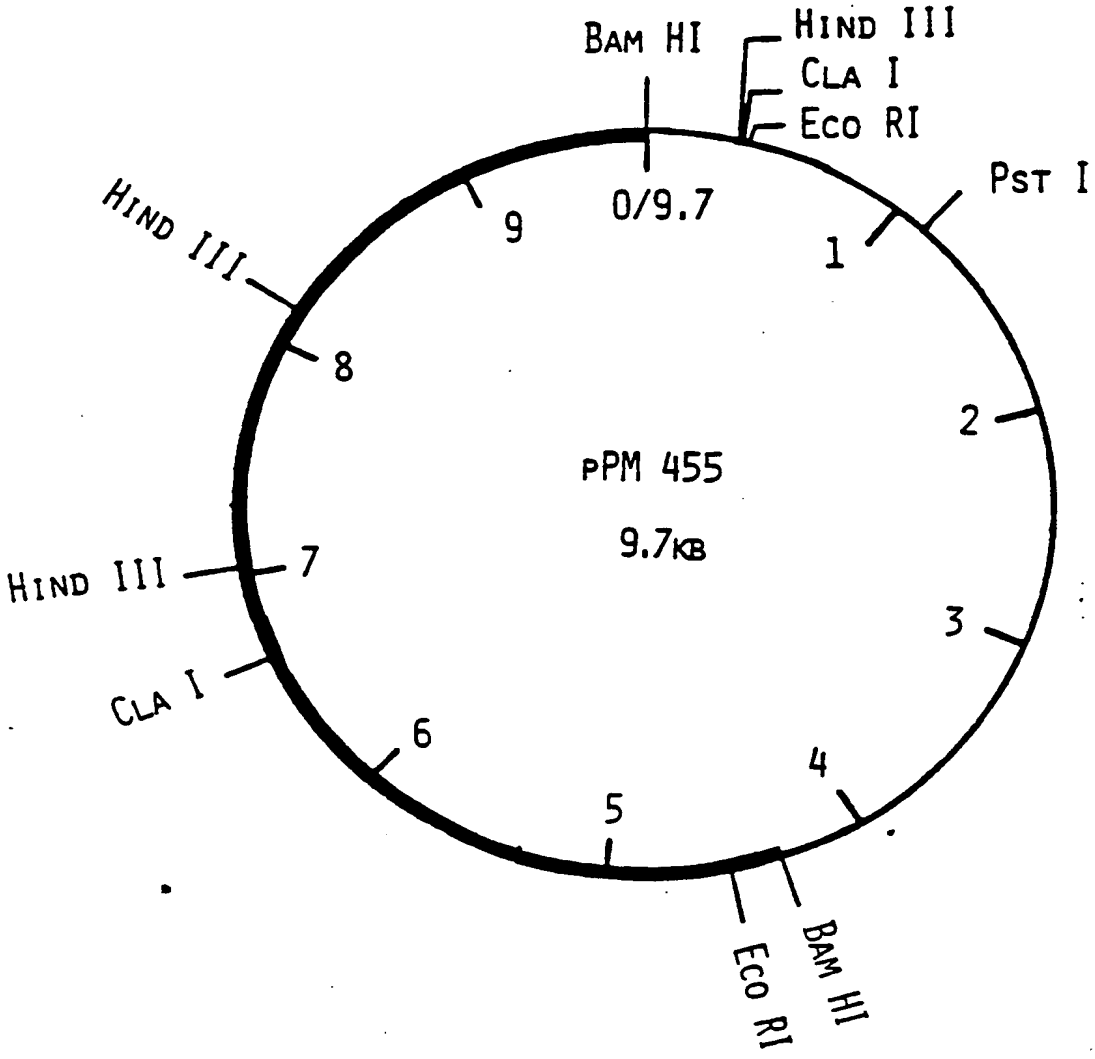


FIGURE 12:

Restriction map of plasmid pPM455. The thick line corresponds to the region of cloned V. cholerae DNA.

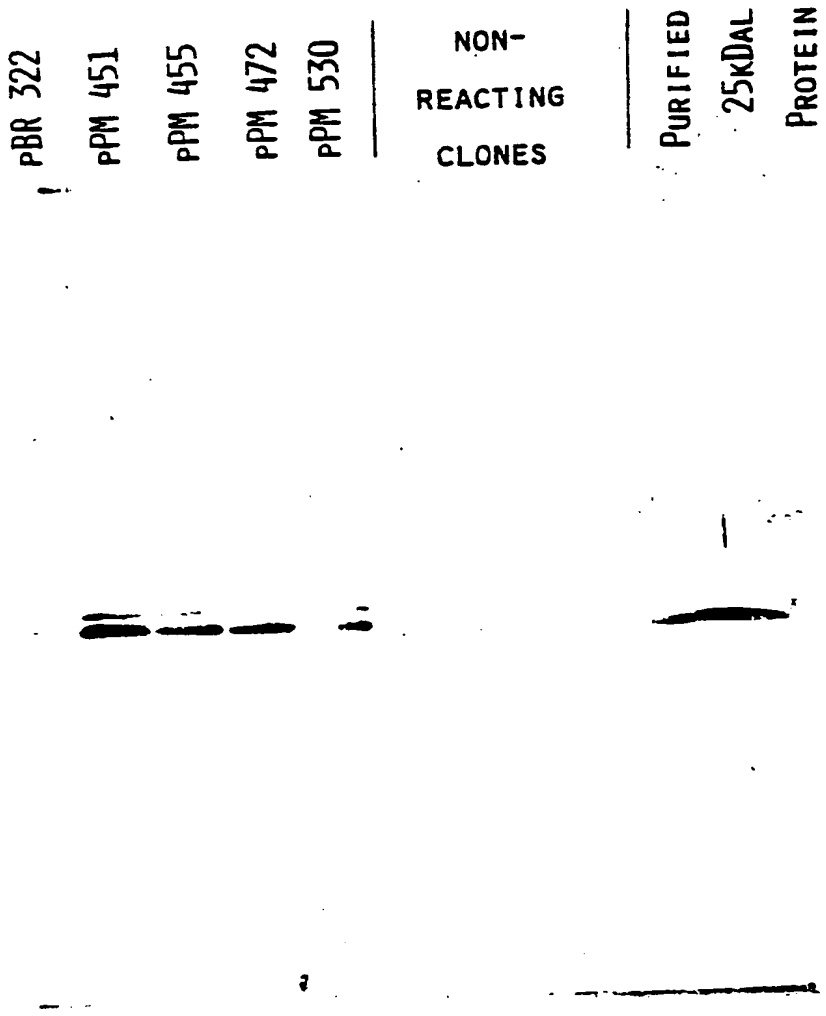


FIGURE 13:

Western blot analysis using anti-25 kDal protein serum with the cell envelopes of E. coli K-12 harbouring the various plasmids. Purified 25 kDal protein (OmpV) is shown for comparison.

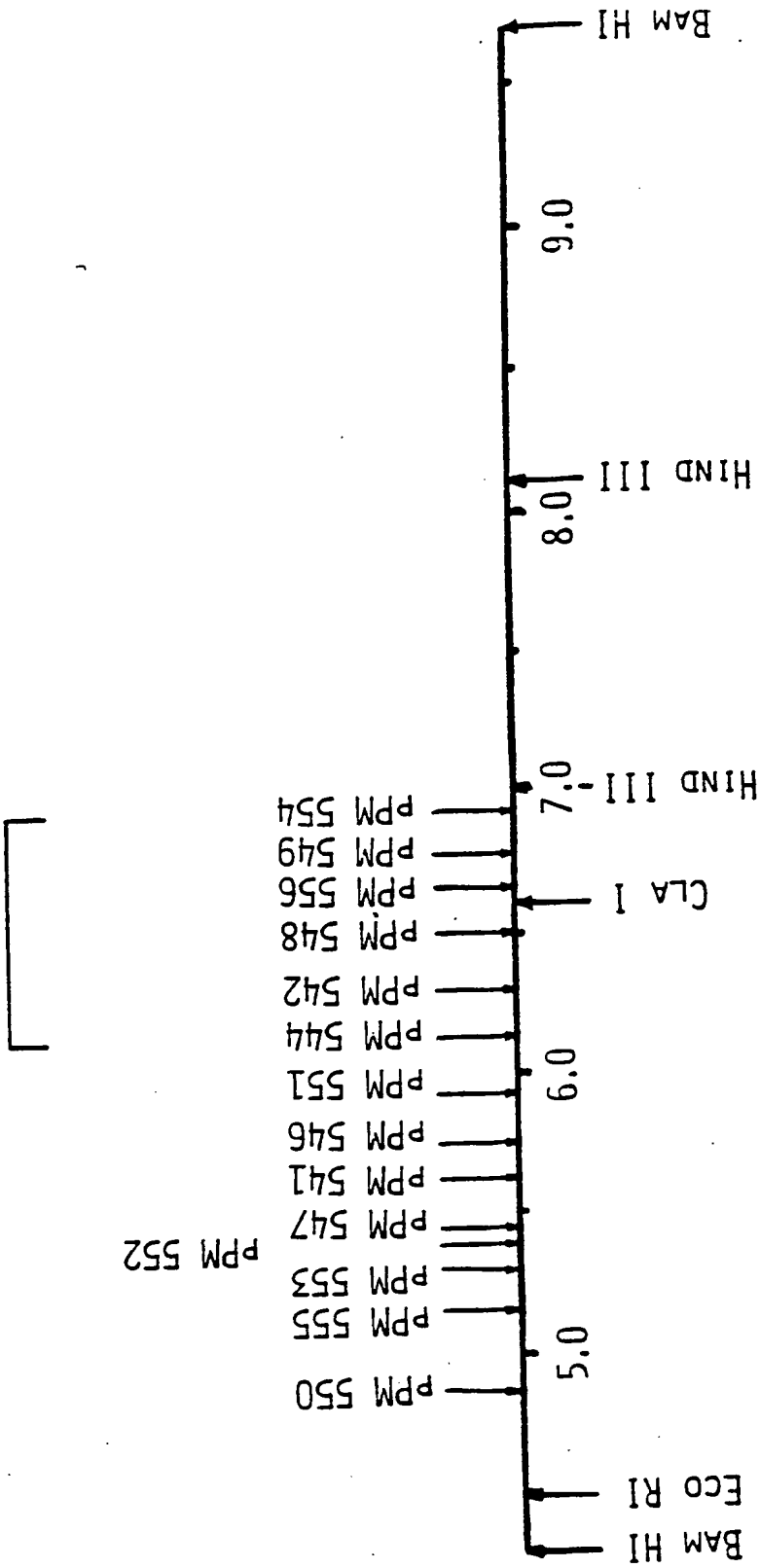


FIGURE 14:

Points of insertion of transposon Tn1725 in the various plasmid derivatives of pPM455, the transposon in plasmids pPM544, pPM542, pPM548, pPM556, pPM549 and pPM554 all eliminate the ability to detect the OmpV protein and lie within the gene.

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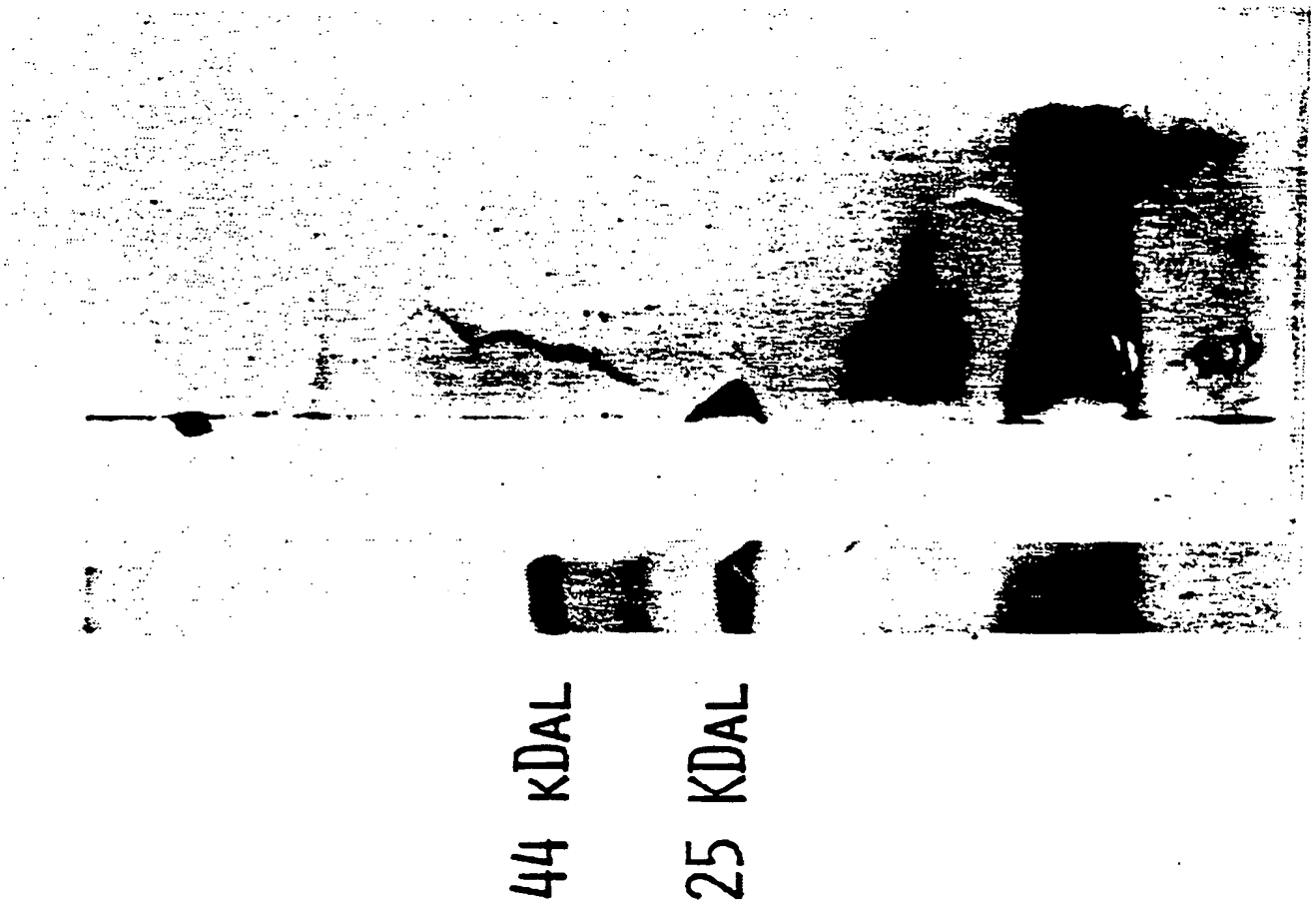


FIGURE 15:

Two dimensional crossed immuno electrophorsis of cell envelope proteins of V. cholerae strain 569B. Cell envelopes were run on SDS polyacrylamide gels in the first dimension, the strip cut out, equilibrated in Triton X-100 and electrophoresed into agarose containing antiserum to V. cholerae 569B.

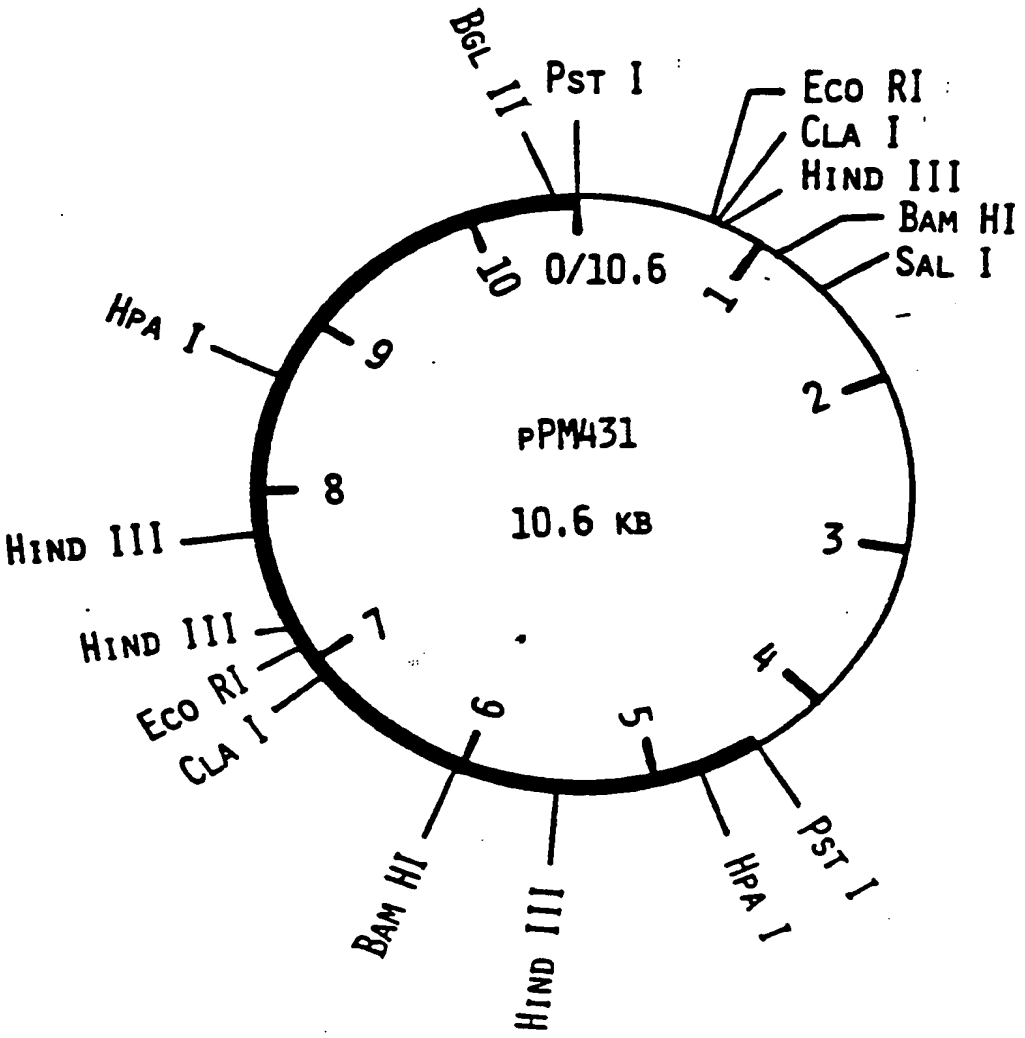


FIGURE 16:

Restriction map of plasmid pPM431. The thick line corresponds to the cloned V. cholerae DNA.

VIBRIO CHOLERAE
017

LE 392

LE 392 [pPM 431]

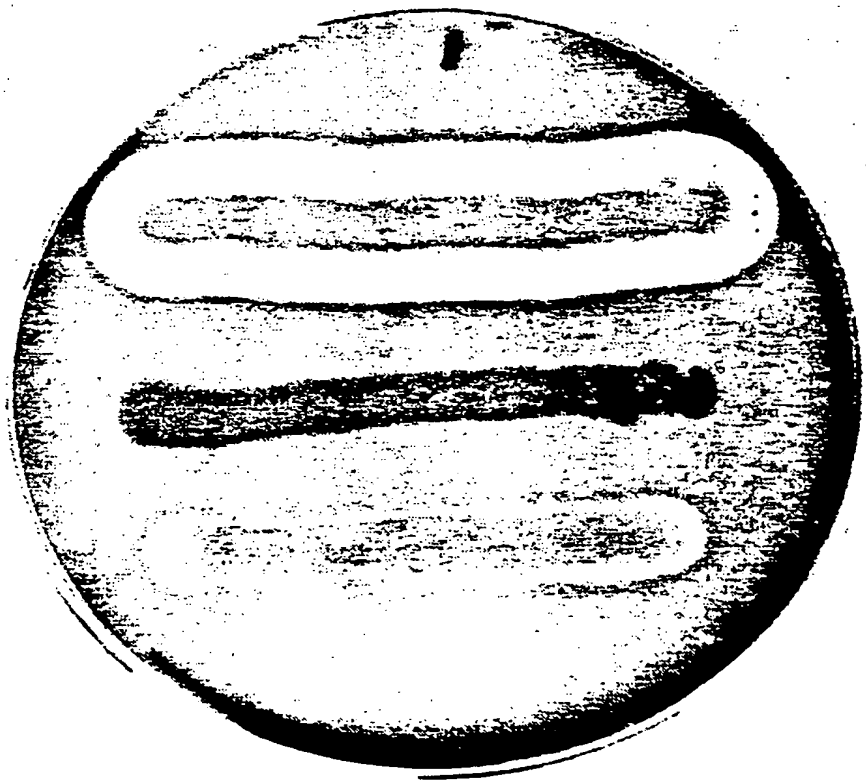


FIGURE 17:

Haemolysin production on nutrient agar containing 5% sheep red blood cells by V. cholerae strain 017 and E. coli K-12 harbouring the plasmids pBR322 (the cloning vector) and pPM431.

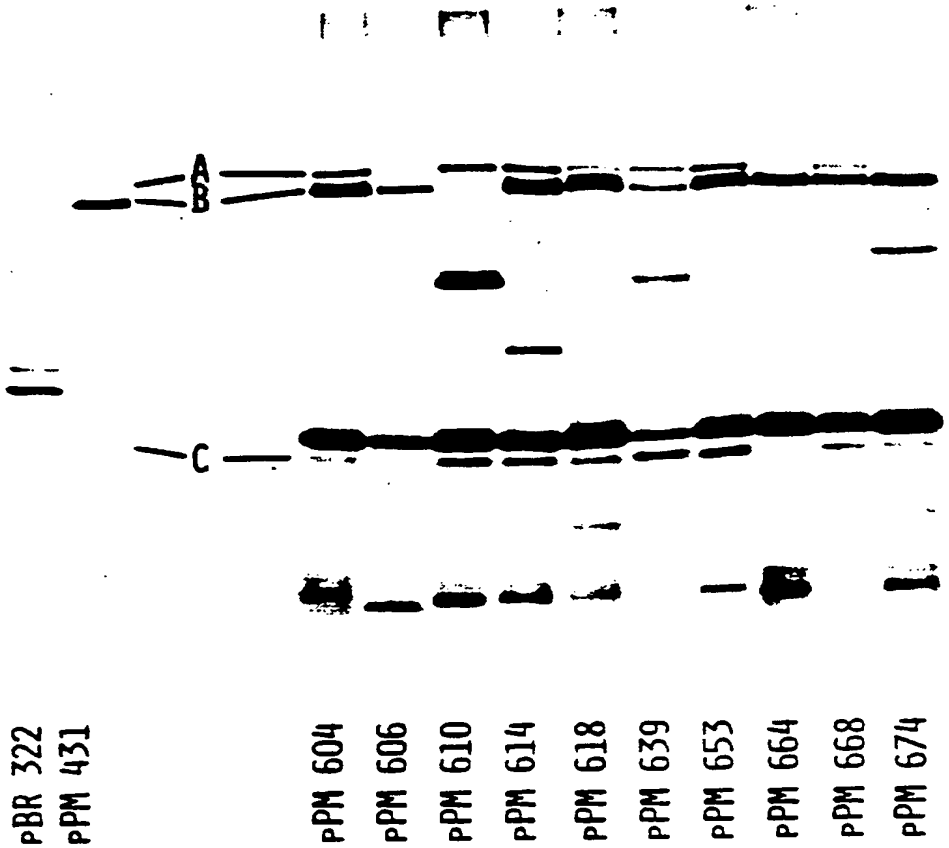


FIGURE 18:

Minicell analysis of transposon Tn1725 derivatives of pPM431. The plasmid encoded proteins were analysed by labelling with (35_S) methionine followed by SDS-polyacrylamide gel electrophoresis and autoradiography.

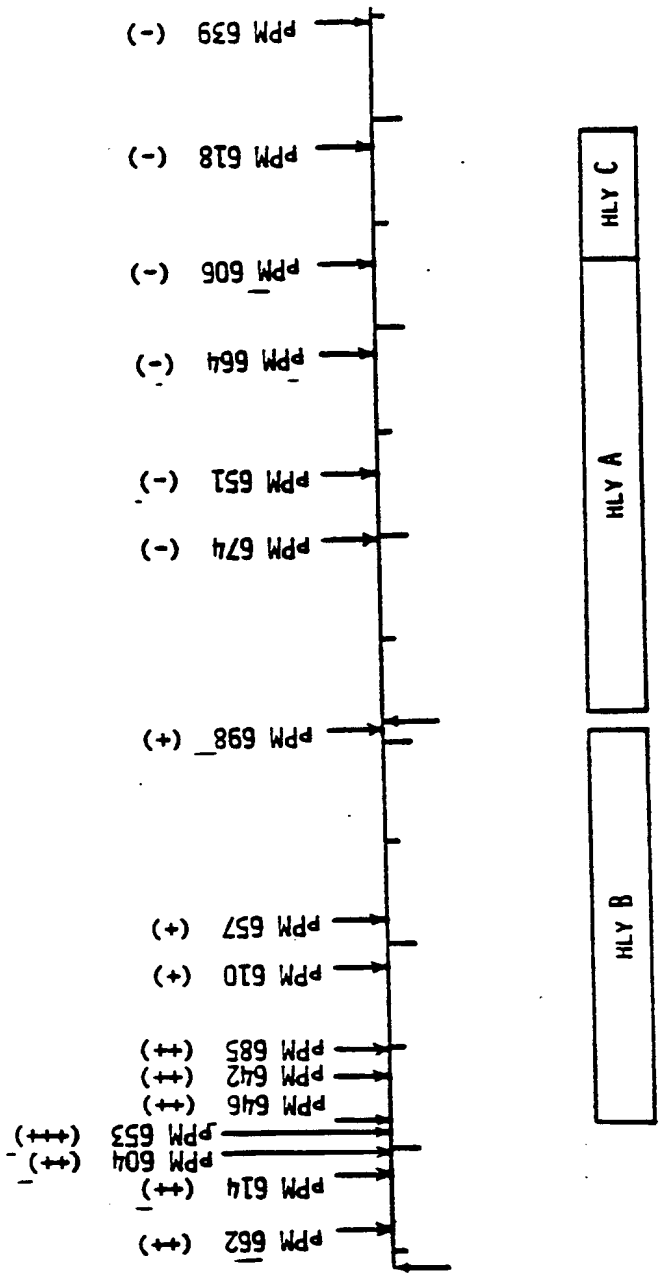


FIGURE 19:

Physical map of the V. cholerae DNA contained within pPM431. The points of insertion of Tn1725 in the various plasmids is shown as is the degree of haemolysis (-, +, ++, +++) compared to pPM431 (+++). The boxes represent the amount and location of DNA encoding each of the proteins.

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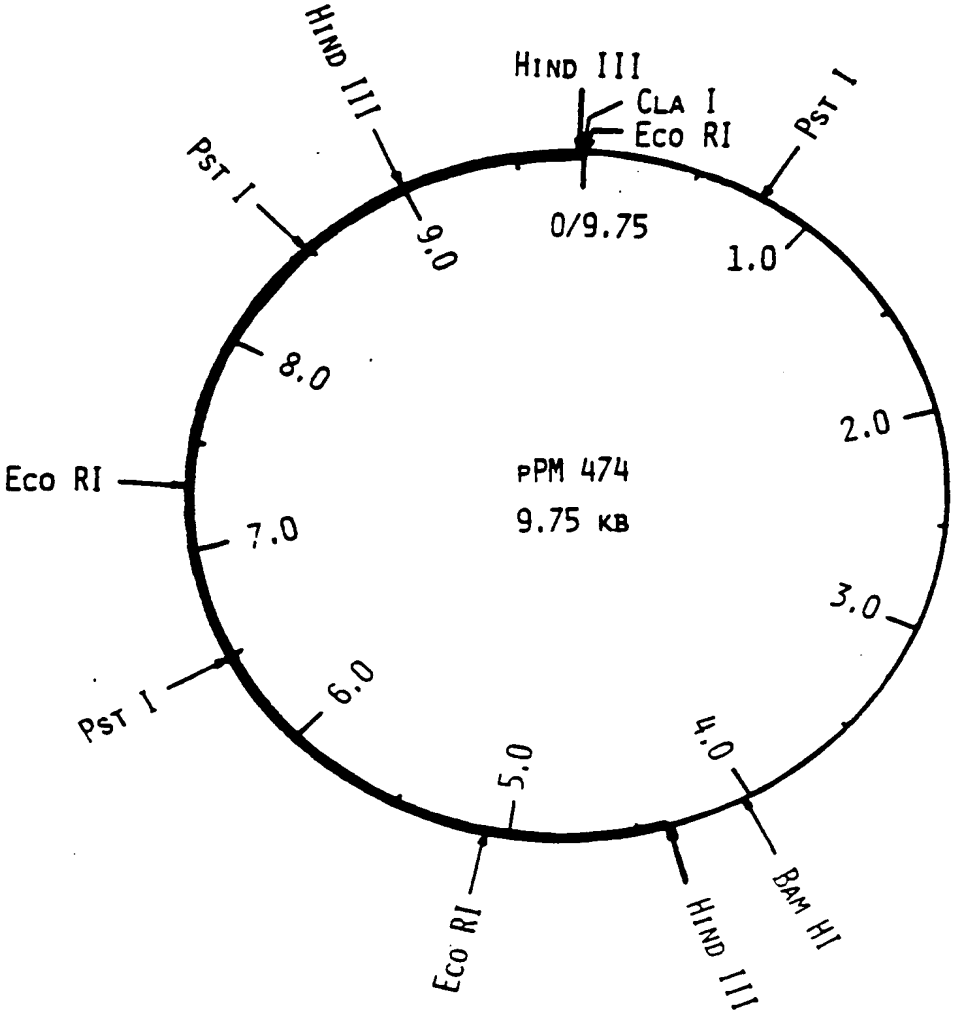


FIGURE 20:

Restriction map of plasmid pPM474. The thick line corresponds to the region of the CFA/II plasmid which has been cloned.

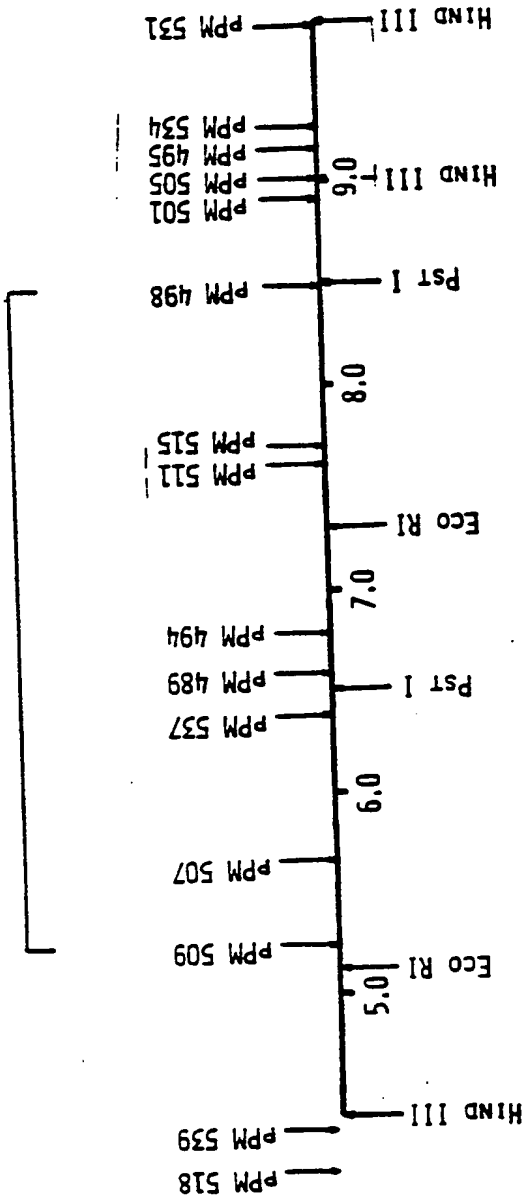


FIGURE 21:

Sites of insertion of transposon Tn1725 into the cloned CFA/II DNA present in pPM474. The coordinates are the same as in Figure 20. Those transposon insertions which have been bracketed have lost the ability to produce CS3.

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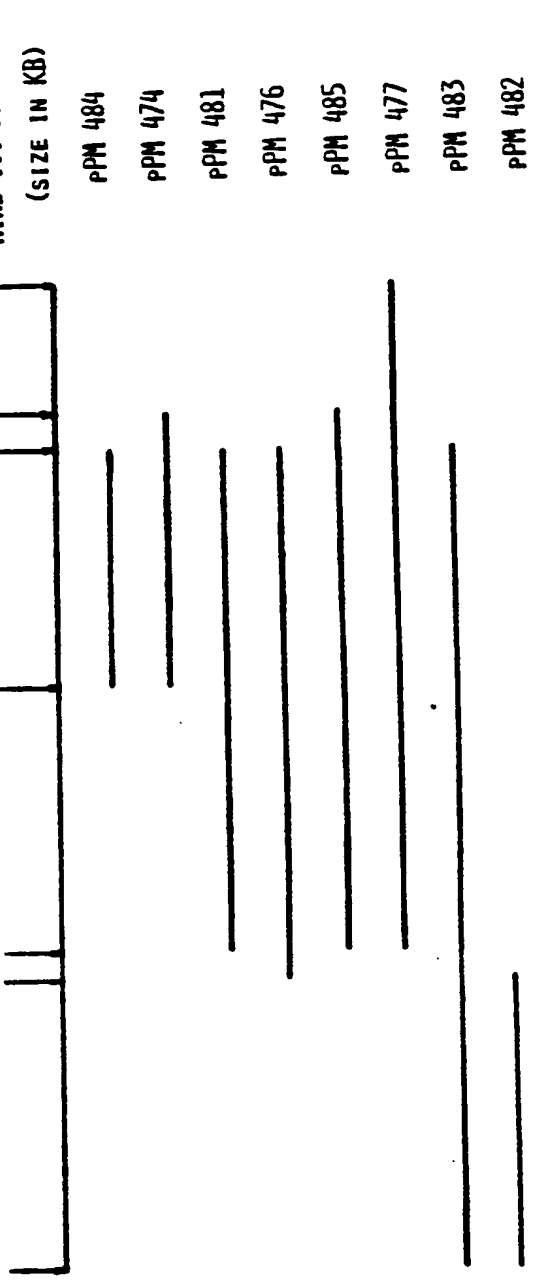


FIGURE 22:

Map of the contiguous HindIII fragments of the CFA/II plasmid which have been cloned into the HindIII site of the plasmid vector pBR322 to give the plasmid derivatives shown.

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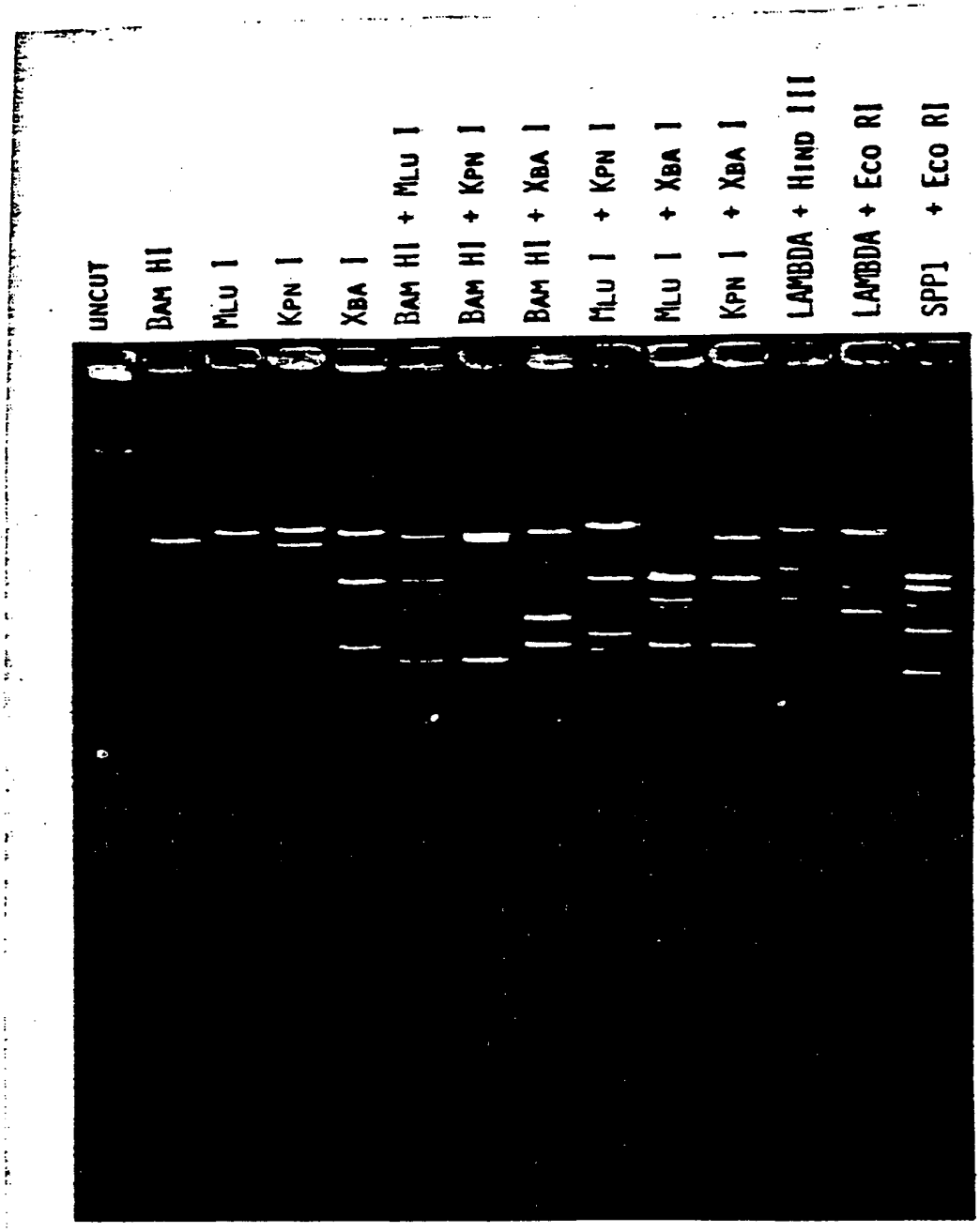


FIGURE 23:

Restriction endonuclease analysis of the DNA of plasmid pPM1001.

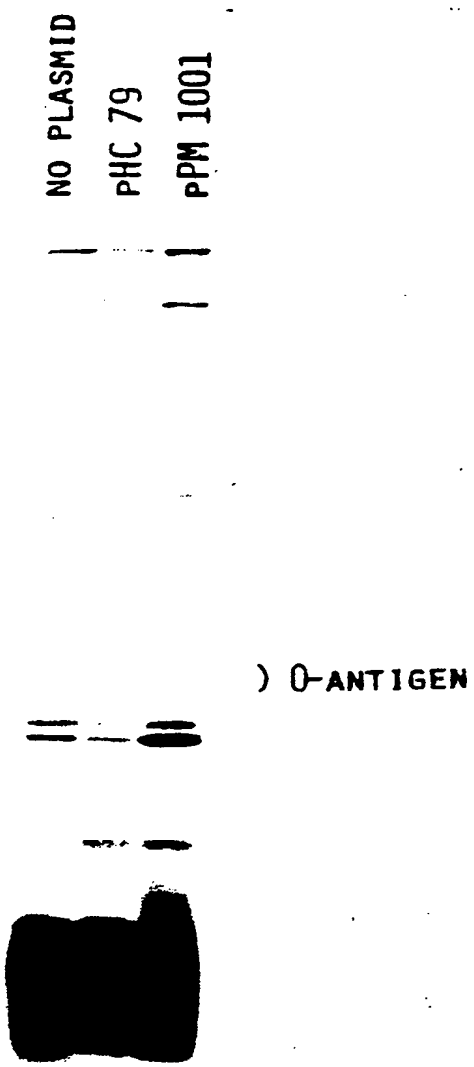


FIGURE 24:

Cell envelopes of E. coli K-12 strain DH1 containing the appropriate plasmids. Cell envelopes were analysed by SDS-polyacrylamide gel electrophoreses followed by silver staining to visualize the bands.

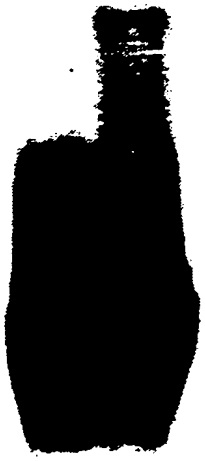


FIGURE 25:

Purified lipopolysaccharide (LPS) of E. coli K-12 strain DH1 with the appropriate plasmids. Purified LPS was analysed by SDS polyacrylamide gel electrophoresis followed by silver staining to visualize the bands.

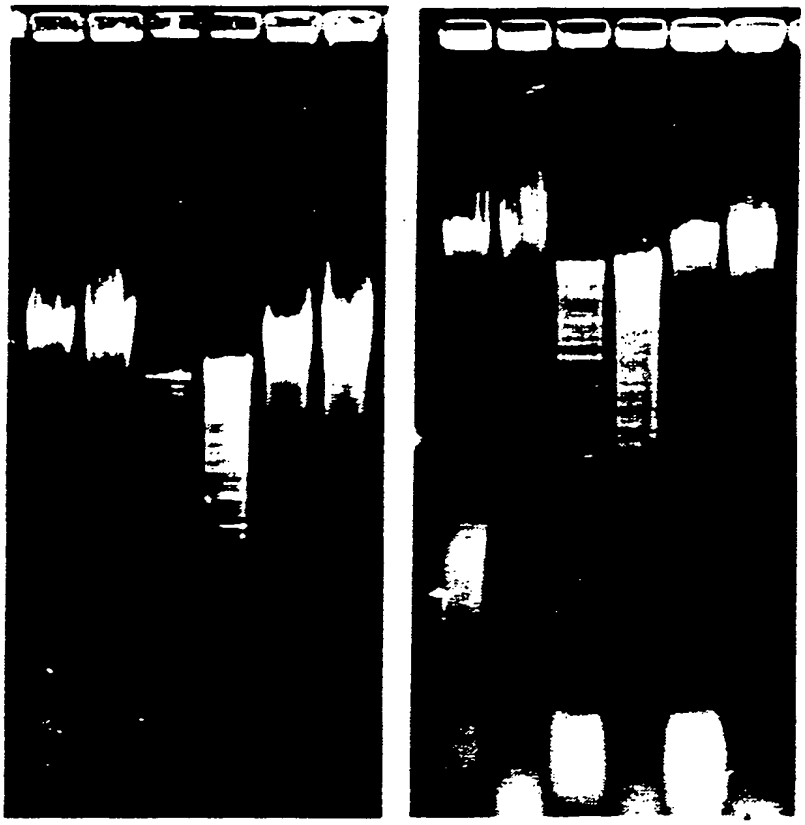


FIGURE 26:

Analysis of chromosomal DNA by electrophoresis in 0.8% agarose in TBE buffer followed by staining with ethidium bromide and visualized using an ultra violet transilluminating light source. Restriction digests and ligations were performed according to the manufacturers' recommendations.

1. Vibrio cholerae 569B DNA uncut
2. Salmonella typhimurium LT2 DNA uncut
3. DNA as in 1 but digested with either Pst1 (left panel) or EcoR1 (right panel)
4. DNA as in 2 but digested with either Pst1 (left panel) or EcoR1 (right panel)
5. DNA as in 3 but ligated
6. DNA as in 4 but ligated.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 85/00015

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 15/00, C12N 1/20, C07G 17/00, A61K 39/06, C12P 19/34 // (C12N 15/00, C12R 1:19)(C12N 15/00, C12R 1:42)(C12N 15/00, C12R 1:63)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C12N 15/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

B10S1S previous data base search

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	AU, A, 83310/82 (CETUS CORPORATION) 16 September 1982 (16.09.82)	(1-8,18)
X	Infect. Immun. 34(3). 1981, 746-750 Formal S.B., et al "CONSTRUCTION OF A POTENTIAL BIVALENT VACCINE STRAIN: INTRODUCTION OF SHIGELLA-SONNEI FORM I ANTIGEN GENES INTO GALE SALMONELLA-TYPH I TY-21A TYPHOID VACCINE STRAIN"	(1,18)
X	Infect. Immun. 46(2). 1984, 564-569 Clements J.D., et al "CONSTRUCTION OF A POTENTIAL LIVE ORAL BIVALENT VACCINE FOR TYPHOID FEVER AND CHOLERA-ESCHERICHIA-COLI RELATED DIARRHEAS"	(1,18)
A	Infect. Immun. 34(3). 1981, 1018-1024 LU Y-S., et al "PROTECTION OF RABBITS AGAINST EXPERIMENTAL PASTEURELLOSIS BY A STREPTOMYCIN-DEPENDANT PASTEURELLA-MULTOCIDA SEROTYPE 3 : A LIVE MUTANT VACCINE"	(1,18)

* Special categories of cited documents: [10]

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 March 1985 (15.03.85)	(07-05-85) 07 MAY 1985
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.W. ASHMAN

Form PCT/ISA/210 (second sheet) (January 1985)

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU **85/00015**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members				
AU 83310/82	DK 1019/82	EP 60129	JP 57171926		
	NZ 199866	WO 8203088			

END OF ANNEX

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